P-306 (TelAviv)

DRAFT: October 4, 1994

PREVENTION OF PROTEIN AGGREGATION VIA MONOCLONAL ANTIBODIES AND GENETICALLY ENGINEERED ANTIBODY FRAGMENTS

ABSTRACT OF THE DISCLOSURE

Start

EXHIBIT K

Fig 1.

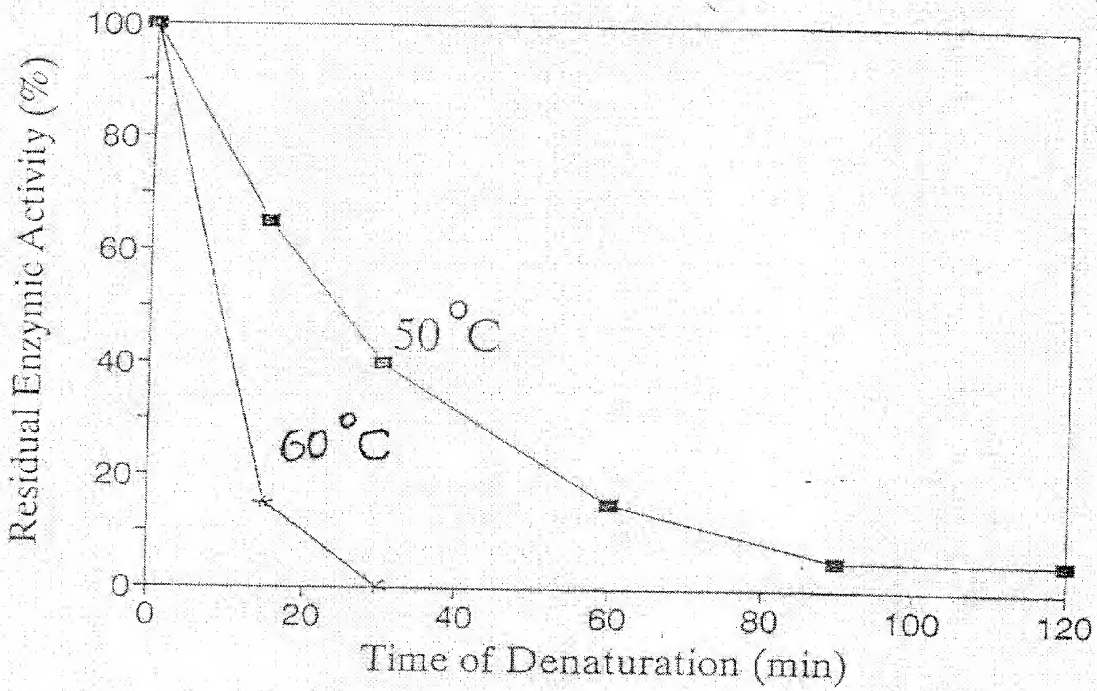

Residual Enzymic Activity (%)
100
80
60
40
20
0
0
20
40
60
80
100
120
50 °C
60 °C
Time of Denaturation (min)

Fig 10

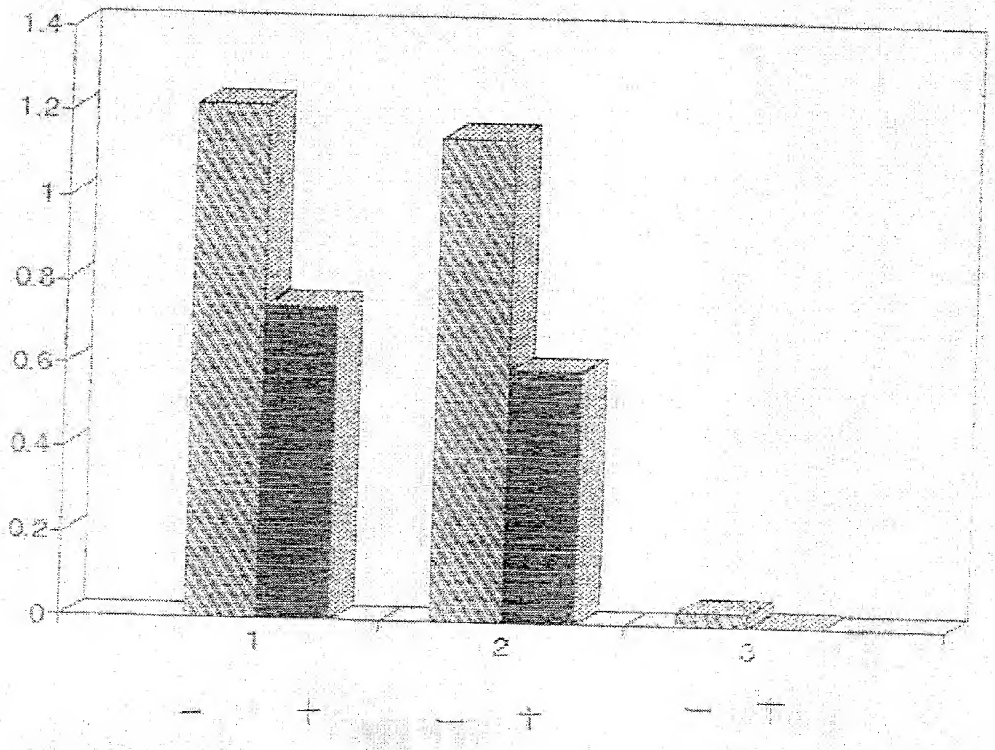

1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
2
3
−
+
−
+
−
+

Fig. 11

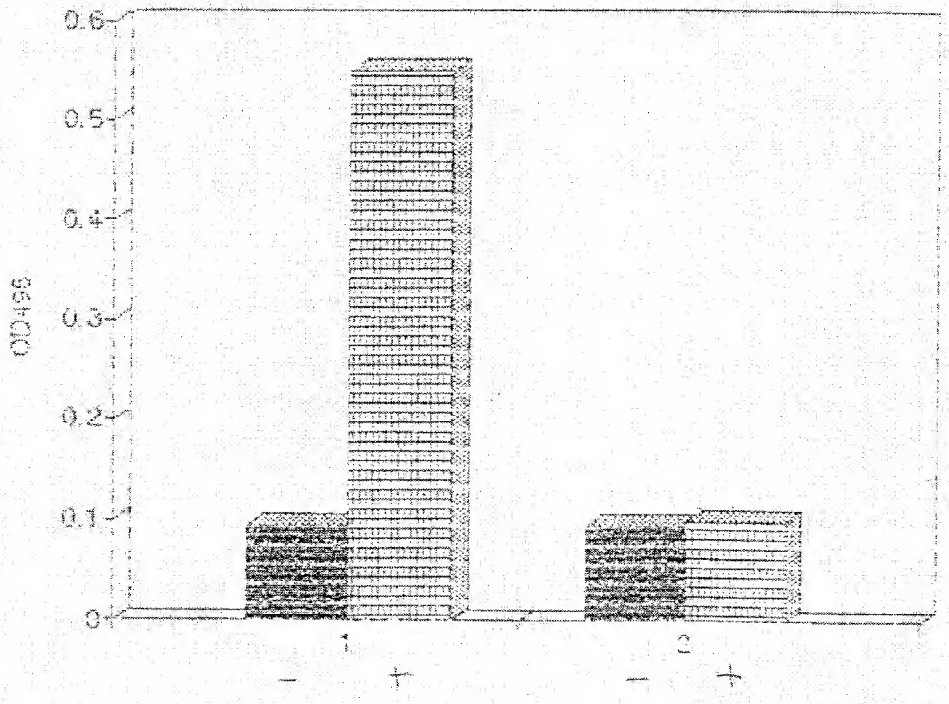


Fig. 12

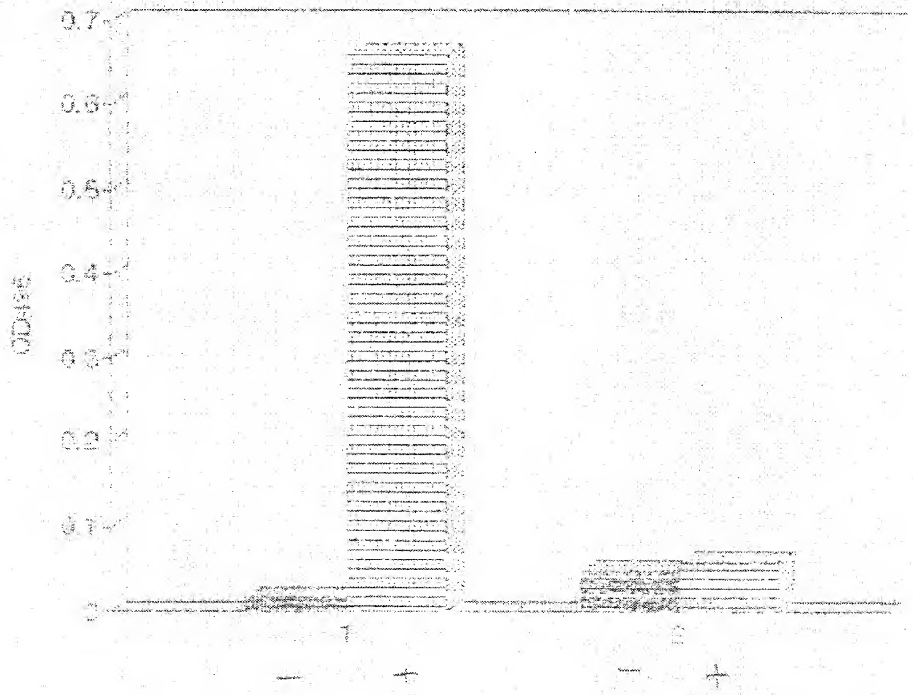
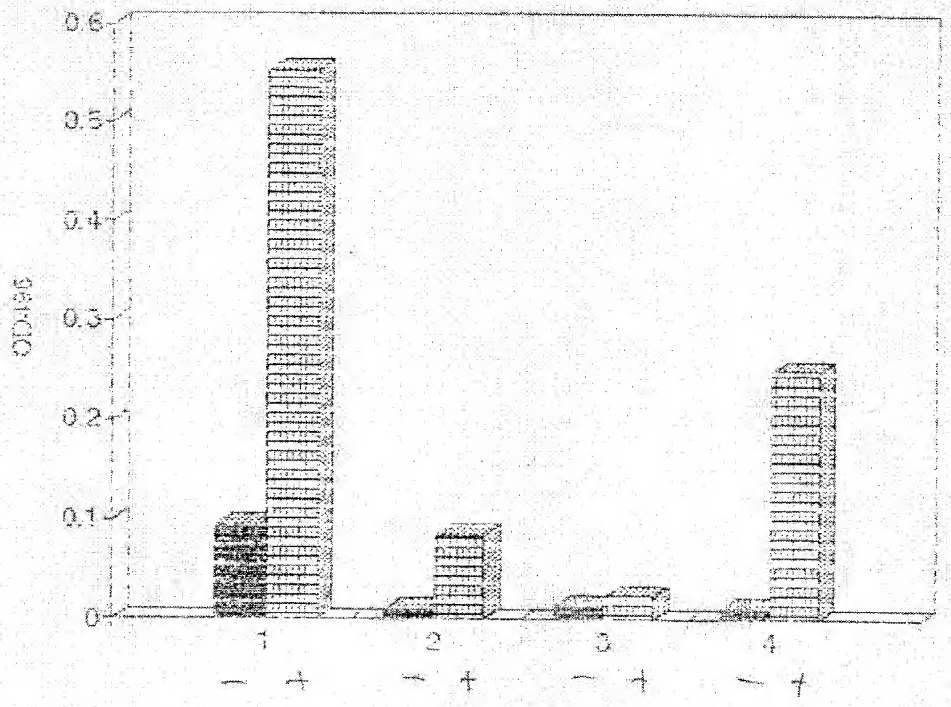


Fig 13.



Legends to Figures 10-13

Figure 10: Prevention of ß-amyloid aggregation by mAb AMY 33, as measured by competitive ELISA.

1. ß-amyloid was incubated for 3 h at 37°C in the absence (-) or presence (+) of mAb.
2. The same experiment in the presence of 50 mM of Heparan sulfate.
3. The same experiment in the presence of Zn^{2+}

Figure 11: Prevention of ß-amyloid aggregation by two different mAbs, AMY 33 (1) and 6F/3D (2) as measured by diect ELISA

(-) - the incubation at 37°C for 3 h in the absence of antibody

(+) - the incubation at 37°C for 3 h in the presence of antibody.

Figure 12: Aggregation of ß-amyloid in the presence of 50 mM heparan sulfate determined by direct ELISA

1. mAb AMY 33
2. mAb 6F/3D

Figure 13: Aggregation of ß-amyloid in the presence of metal chloride solutions in the presence (+) and absence (-) of mAb AMY 33 determined by direct ELISA

2. In the presence of Zn^{2+}

3. In the pesence of Al^{3+}

4. In the pesence of Ca^{2+}

PREVENTION OF PROTEIN AGGREGATION VIA MONOCLONAL ANTIBODIES AND GENETICALLY ENGINEERED ANTIBODY FRAGMENTS

BACKGROUND OF THE INVENTION

TECHNICAL FIELD

The present invention relates to the use of monoclonal antibodies and genetically engineered antibody fragments for the prevention of protein aggregation.

BACKGROUND ART

When proteins are synthesized they generally must fold and assemble into the complete three dimensional form to be active. Initially, it was thought that proper folding was inherent in the amino acid sequence. Recent work has shown that additional proteins, now referred to as molecular chaperones, are required to mediate the folding process or unregulated aggregation of the polypeptides will occur preventing the formation of functional proteins. [Welch, 1993; Ellis et al, 1979] However, despite the existence of chaperones, aggregation

of protein still occurs *in vivo* and can contribute to, or cause, various disease states.

Other factors must contribute to the occurrence of aggregation. These factors can include mutations which do not allow the naturally occurring chaperones to function or to function only with low efficiency [Wetzel, 1994]. It would be useful to be able to replace the activity of the chaperones where necessary.

Protein aggregation is of major importance in biotechnology for the *in vitro* production of recombinant proteins. *In vitro* aggregation limits the protein stability, solubility and yields in production of recombinant proteins. In cells during production of recombinant proteins, aggregation is a major impediment of recombinant proteins leading to formation of inclusion bodies in the host cells. [DeYoung, et al, 1993; Wetzel, 1994; Vandenbroeck et al, 1993]

Further, *in vivo* protein aggregation or precipitation is the cause, or an associated pathological symptom, in amyloid diseases such as Down's syndrome, Alzheimer's disease, diabetes and/or cataracts, and in other disorders. [DeYoung et al, 1993; Selkoe, 1991; Wetzel, 1994]

Several peptides including β-amyloid, have been shown to spontaneously self-associate, or aggregate, into linear, unbranched fibrils in serum or in isotonic saline [Oakley et al, 1981; Kastin et al, 1984; Selkoe, 1991]. Iron, zinc, chromium or aluminum can participate in this aggregation. ~~[reference]~~ Beyreuther 1992.

Molecular chaperones were initially recognized as stress proteins produced in cells requiring repair. In particular, studies of heat shock on enzymes led the way to the discovery of molecular chaperones that function not only during cellular stress but normally to produce properly folded proteins. The heat shock model is still one of the models of choice in studying molecular chaperones. [Welch, 1993; Ellis et al, 1991].

Molecular chaperones are a ubiquitous family of proteins that mediate the post-translational folding and assembly of other unrelated proteins into oligomeric structures. They are further defined as molecules whose functions are to prevent the formation of incorrect structures and to disrupt any that form. The chaperones non-covalently bind to the interactive surface of the protein. This binding is reversed under circumstances that favor the

formation of the correct structure by folding. {Ellis et al, 19??} Chaperones have not been shown to be specific for only one protein but rather act on families of proteins which have the same stoichiometric requirements, i.e specific domains are recognized by chaperones. [INVENTOR: is this a correct statement - you have indicated in your disclosure that chaperones are not specific. Please provide a further argument for your statement that chaperones are not specific]

Further uses and descriptions of molecular chaperones are set forth in PCT published international patent application 93/11248, 93/13200, 94/08012 and 94/11513 incorporated herein by reference and in particular 94/08012 page 2 line 20 through page 5, line 14.

PCT published international patent application 93/11248 discloses the use of a chaperone in cell culture to promote efficient production of protein in transformed cells by co-expression of the chaperone molecule. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 93/13200 discloses the use of a chaperone in a purification step for a recombinant protein isolated from a cell culture and also a fusion protein of the chaperone and recombinant protein. This disclosure also does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/08012 discloses the use of a chaperone in cell culture to promote increased secretion of an overexpressed gene product in a host cell. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically.

PCT published international patent application 94/11513 discloses the use of a vector containing a molecular chaperone for treating neoplasms. This disclosure does not provide specificity as to which proteins are protected except through co-expression with the wanted protein nor does it provide information on how to use chaperones therapeutically to treat

diseases or syndromes which involve protein aggregation.

[INVENTOR: please review published applications (copies enclosed) and elaborate in further detail on how they are different from your application, particularly WO 94/11513.]

Recent reports suggest that monoclonal antibodies (mAb) can act as chaperones. The feasibility of using monoclonal antibodies to assist in the *in vitro* refolding process of guanidine-denatured S-protein was reported recently. [Carlson and Yarmush, 1992]. Previously, Blond and Goldberg [1987] used monoclonal antibodies as a tool in the identification and characterization of folding steps that involve the appearance of local native-like structures in B_2 subunit of tryptophan-synthase. Since the mAb is epitope specific, the use of mAb provides more specificity than molecular chaperones. mAbs can be sought and engineered [Haber, 1992] that bind to the particular epitope in the protein of interest that is involved in the folding process. [INVENTOR: mAb are only sequence or epitope specific - if the sequence exists in any protein

it will bind. How is this different in the degree of specificity from molecular chaperones (domain specific) since we cannot claim that a mAb is specific for only one protein unless we show that it binds to a sequence unique to that protein?]

Aggregated amyloid β-protein (BA^4) is a major constituent of the abnormal extracellular amyloid plaque that characterizes the brains of victims of Alzheimer's disease (AD). [~~Greenberg, et al, 19??~~, INVENTOR please provide complete citation] *In vitro* studies have shown that some of the metal ions found in biological systems, i.e. Fe, Al and Zn, can accelerate the aggregation process dramatically.[reference?] [INVENTOR: is there a reference showing metal-induced aggregation *in vivo*?] If the interaction between the metal ion and the β-amyloid can be interrupted or prevented, then metal-induced aggregation can be reduced or eliminated. However, just binding a mAb at this site might prevent the metal-induced aggregation but would not allow normal functioning of the protein.

It would therefore be useful to develop the appropriate mAb with chaperone characteristics directed to the appropriate

epitope on the β-amyloid molecule in order to prevent the accelerated metal-induced aggregation without interfering with β-amyloid function. — No function

Further, it would be particularly useful to develop a mAb that prevents the aggregation of enzymes *in vivo* but that still allows the enzymes to function.

Still further, it is not always possible to isolate the appropriate chaperone for preventing aggregation of a molecule and to utilize it as a therapeutic. The availability of engineering and selecting mAbs and delivery systems for mAb makes it useful to develop specific mAb to serve as therapeutic chaperones. [INVENTOR: please review the above, particularly the last several paragraphs. Do these paragraphs adequately and accurately state the problem to be solved?]

SUMMARY OF THE INVENTION AND ADVANTAGES

According to the present invention, a method is provided of selecting monoclonal antibodies that can ~~function as chaperones~~. These monoclonal chaperone antibodies are able to bind to a target molecule epitope with a high

binding constant and must be non-inhibitory to biological activity of the target molecule.

The present invention further provides a method of treating a protein aggregation disease by creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human monoclonal antibody that binds to a target molecule, an aggregating protein, and which prevents aggregation and allows biological activity of the target molecule.

BRIEF DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention will be readily appreciated as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

FIGURE 1 is a graph of the loss of enzymic activity of Carboxypeptidase A after incubation at 50°C (■) and 60°C (x) for different periods of time and measuring residual enzymic activity by esterase substrate; [INVENTOR: please

provide better copy of Figure in which 60° curve shows]

FIGURE 2 is a bar graph of the dependence of refolding of Carboxypeptidase A on denaturation degree of the enzyme, the enzymatic activity of the enzyme and its immunocomplexes determined using esterase substrate, each experimental result represents the average of at least three independent measurements;

FIGURE 3 is a bar graph of the refolding of Carboxypeptidase A in the presence of the monoclonal antibodies CP 10, CP 9, CP 8 and CP 32; Figure 4 is a graph of the time dependence of refolding process of Carboxypeptidase A with monoclonal antibodies CP 10 (+) and CP 9 (*) in the presence of PBS (■);

Figure 5 is a bar graph of the additive effect of pairs of mAbs on CPA refolding, mAbs pairs of (CP 10 + CP 9), (CP 10 + CP 8), (CP 10 + CP 32) were measured;

Figure 6 is a graph of carboxypeptidase A, showing that at 50°C in the presence of increasing amounts of specific monoclonal antibody enzymatic activity is restored;

[INVENTOR: please elaborate as in your handwritten notes, we are not sure that we transcribed your notes correctly.]

Figures 7A and B are bar graphs of esterase activity, in controls (-mAb) CPA aggregates at 50°C and in the presence of increasing amounts of Zn^{2+}, addition of monoclonal antibody to the CPA prevent aggregation of the enzyme (+mAb), the effect is dependent on the molar ratios antibody/enzyme (A) small amount of Ab, (B) 5-times more AB to enzyme (w/w);

Figure 8 is a bar graph of control experiments in the presence of an unrelated antibodies (IgG) under the same experimental conditions; and

Figure 9 is a photomicrograph of vials showing a macromolecular view of prevention effect of monoclonal antibodies on aggregation of CPA, vial 1 is CPA alone after incubation for 1 hour at 50°C, vial 2 shows a CPA aggregation in the presence of zinc after incubation for 1 hour at 50°C, vial 3 is CPA + mAb + Zn after incubation for 1 hour at 50°C.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention provides a method of selecting monoclonal antibodies that can ~~function as chaperones and yet not inhibit~~ prevent aggregation

bioactivity. These monoclonal chaperone antibodies are able to bind to a target molecule epitope with a high binding constant and must be non-inhibitory to biological activity of the target molecule. The method includes culturing an appropriate host cell transformed with DNA encoding the target molecule. The host cell chosen will express the target molecule in aggregated form. Examples of such cells are set forth in PCT published international patent application 93/11248, 93/13200 and 94/08012. Alternatively the target molecule can be purchased from commercial sources.

The expressed target molecule is recovered and denatured. The denatured target molecule is mixed with the chaperone monoclonal antibody generally as set forth in PCT pending application 93/13200. It is then determined if the mixture produces nonaggregated target molecules that are bioactive even in the presence of and bound to the monoclonal antibody.

Bioactivity is tested as is appropriate for the target molecule. For example, enzymatic activity of the target molecule for its substrate can be measured. Assays which measure *in vitro* enzymatic bioactivity are well known to those skilled in the art.

CRITERIA FOR THE SELECTION OF MONOCLONAL ANTIBODIES WHICH ACT AS CHAPERONES IN THE PREVENTION OF PROTEIN AGGREGATION

1. High binding constant towards the antigen.
2. Mabs are non-inhibitory to biological activity of the antigen.
3. Mabs bind to strategic locations on the antigen molecule - regions responsible for folding, aggregation, etc.
4. Mabs did not show immune cross reactivity under conditions employed with other proteins from the proximity of the target antigens.
5. Mabs prevent aggregation of their antigen exposed to aggregation conditions such as self-aggregation, temperature, pH or interaction with other aggregation agents.
6. Two or more mAbs can be used concurrently to increase their chaperone-like effect if the epitopes are not overlapped or interfer with eachother.

In the preferred embodiment of the method, the target molecule is β-amyloid and the monoclonal antibody is an anti-β-amyloid monoclonal. The method has also been demonstrated with carboxypeptidase A as set forth in the Examples hereinbelow.

Other peptides or proteins with evidence of self aggregation can also be used in the present invention such as amylin [Edwards and Morley, 1992]; bombesin, caerulein, cholecystokinin octapeptide, eledoisin, gastrin-related pentapeptide, gastrin tetrapeptide, somatostatin (reduced), substance P [Oakley, et al, 1981]; and peptide, luteinizing hormone releasing hormone, somatostatin N-Tyr [Kastin et al, 1984].

[INVENTOR: please comment and elaborate on the general method of selecting monoclonal antibodies for use in your invention. Does the above adequately describe how such a selection would be made? Under the rules of the Patent Office we must provide as detailed a description as possible. Where ever possible please reference if we have not done so.]

Once an appropriate monoclonal chaperone antibody is found or engineered, the present invention provides for its use therapeutically to prevent or reduce protein aggregation *in vivo*. In the preferred embodiment, the prevention of β-amyloid aggregation is undertaken.

The method of treating a protein aggregation disease includes the steps of preparing [Haber, 1992; Harlow & Lane, 1988] or selecting a human monoclonal antibody that binds to an aggregating protein which is the cause of a disease and which prevents aggregation and yet allows the protein to be bioactive which is referred to as a monoclonal chaperon antibody. An expression vector is created comprising nucleic acid including a sequence which encodes in expressible form the human monoclonal chaperone antibody. The expression vector is then delivered to the patient.

In the preferred embodiment the human monoclonal antibody that binds to an aggregating protein and which prevents aggregation is an anti-β-amyloid and is designated AMY-33 [SOURCE?]

which recognizes amino acids 1-28 of β-amyloid (SEQ ID No:1). [INVENTOR: we will need to provide this amino acid sequence to the Patent Office. Please provide an ASCII file of the amino acid sequence so that we can submit it to the Patent Office.]

Work by Duevas et al. [1994] and Marasco et al [1993] have shown that single chain monoclonal antibodies are efficient for intracellular expression in eukaryotic cells. The single chain monoclonal antibody is composed of an immunoglobulin heavy chain leader sequence and heavy and light chain variable regions that are joined by an interchain linker. Marasco et al [1993] have shown that such antibodies are not toxic to the cells and function when expressed in the cell.

The production of expression vectors is well known to those skilled in the art. In a preferred embodiment, the expression vector is constructed using the methodology as set forth by Duevas et al. [1994], PCT pending application 94/11513. Methods not explicitly set forth are performed as generally set forth in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor Laboratory, New York (1992),

and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989).

Such vectors are known or can be constructed by those skilled in the art and should contain all expression elements necessary to achieve the desired transcription of the sequences. Other beneficial characteristics can also be contained within the vectors such as mechanisms for recovery of the nucleic acids in a different form. Phagemids are a specific example of such beneficial vectors because they can be used either as plasmids or as bacteriophage vectors. Examples of other vectors include viruses such as bacteriophages, baculoviruses and retroviruses, DNA viruses, cosmids, plasmids, liposomes and other recombination vectors. The vectors can also contain elements for use in either procaryotic or eucaryotic host systems. One of ordinary skill in the art will know which host systems are compatible with a particular vector.

The expression vector can be a virus. Further the virus can be an RNA virus such as a

disabled retro virus or a retroviral shuttle vector. The expression vector can also be vaccinia virus or an adenovirus. The expression vector can also be a plasmid. In a preferred embodiment wherein β-amyloid in the targeted molecule the expression vector is selected that is known to target the central nervous system.

In the present invention, the expression vector for use as a therapeutic agent comprises a nucleic acid including at least one sequence which encodes in expressible form a human monoclonal antibody, which human monoclonal antibody binds to an aggregating protein that is the cause of a disease and which prevents aggregation but does not interfere with bioactivity. In a preferred embodiment the expression vector includes the sequence for a human monoclonal antibody that is an anti-β-amyloid monoclonal antibody. In a further preferred embodiment, the expression vector includes the sequence for a single chain monoclonal antibody.

A specific example of DNA viral vector for introducing and expressing recombinant sequences is the adenovirus derived vector Adenop53TK. This vector expresses a herpes virus thymidine kinase (TK) gene for either positive or

negative selection and an expression cassette for desired recombinant sequences. This vector can be used to infect cells that have an adenovirus receptor. This vector as well as others that exhibit similar desired functions can be used to treat a mixed population of cells and can include, for example, an *in vitro* or *ex vivo* culture of cells, a tissue or a human subject.

Additional features can be added to the vector to ensure its safety and/or enhance its therapeutic efficacy. Such features include, for example, markers that can be used to negatively select against cells infected with the recombinant virus. An example of such a negative selection marker is the TK gene described above that confers sensitivity to the antibiotic gancyclovir. Negative selection is therefore a means by which infection can be controlled because it provides inducible suicide through the addition of antibiotic. Such protection ensures that if, for example, mutations arise that produce altered forms of the viral vector or recombinant sequence, cellular transformation will not occur. Features that limit expression to particular cell types can also be included. Such features include, for example, promoter and

regulatory elements that are specific for the desired cell type.

In addition, recombinant viral vectors are useful for *in vivo* expression of a desired nucleic acid because they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural

specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. The vector to be used in the methods of the invention will depend on desired cell type to be targeted and will be known to those skilled in the art. For example, if breast cancer is to be treated then a vector specific for such epithelial cells would be used. Likewise, if diseases or pathological conditions of the hematopoietic system are to be treated, then a viral vector that is specific for blood cells and their precursors, preferably for the specific type of hematopoietic cell, would be used.

Retroviral vectors can be constructed to function either as infectious particles or to undergo only a single initial round of infection. In the former case, the genome of the virus is modified so that it maintains all the necessary genes, regulatory sequences and packaging signals to synthesize new viral proteins and RNA. Once these molecules are synthesized, the host cell packages the RNA into new viral particles which are capable of undergoing further rounds of infection. The vector's genome is also engineered to encode and express the desired recombinant gene. In the

case of non-infectious viral vectors, the vector genome is usually mutated to destroy the viral packaging signal that is required to encapsulate the RNA into viral particles. Without such a signal, any particles that are formed will not contain a genome and therefore cannot proceed through subsequent rounds of infection. The specific type of vector will depend upon the intended application. The actual vectors are also known and readily available within the art or can be constructed by one skilled in the art using well-known methodology.

[INVENTOR: please correct as needed and elaborate where possible on the above.]

The expression vector may be administered to mammals, including humans, by any route appropriate to the condition being treated and in several ways. Suitable routes include oral, rectal, nasal, topical, vaginal and parenteral. It will be appreciated that the preferred route may vary with, for example, the condition of the recipient and the type of treatment envisaged.

If viral vectors are used, for example, the procedure can take advantage of their target specificity and consequently, do not have to be administered locally at the diseased site. However, local administration can provide a quicker and more effective treatment, administration can also be performed by, for example, intravenous or subcutaneous injection into the subject. Injection of the viral vectors into a spinal fluid can also be used as a mode of administration, especially in the case of neurodegenerative diseases. Following injection, the viral vectors will circulate until they recognize host cells with the appropriate target specificity for infection.

The vectors can be introduced into cells or tissues by any one of a variety of known methods within the art. Such methods can be found described in Sambrook et al. and Ausubel et al., and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. Introduction of nucleic acids by infection offers several advantages over the other listed methods. Higher efficiency can be obtained due to their infectious nature. Moreover, viruses are very specialized and

typically infect and propagate in specific cell types. Thus, their natural specificity can be used to target the vectors to specific cell types *in vivo* or within a tissue or mixed culture of cells. Viral vectors can also be modified with specific receptors or ligands to alter target specificity through receptor mediated events.

An alternate mode of administration of the vector can be by direct inoculation locally at the site of the disease or pathological condition or by inoculation into the vascular system supplying the site with nutrients. Local administration is advantageous because there is no dilution effect and, therefore, a smaller dose is required to achieve expression in a majority of the targeted cells. Additionally, local inoculation can alleviate the targeting requirement required with other forms of administration since a vector can be used that infects all cells in the inoculated area. If expression is desired in only a specific subset of cells within the inoculated area, then promoter and regulatory elements that are specific for the desired subset can be used to accomplish this goal. Such non-targeting vectors can be, for example, viral vectors, viral genome, plasmids, phagemids and the like. Transfection

vehicles such as liposomes can also be used to introduce the non-viral vectors described above into recipient cells within the inoculated area. Such transfection vehicles are known by one skilled within the art.

The expression vector of the present invention may be administered to the patient alone or in combination with liposomes or other delivery molecules. The expression vector is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, and other factors known to medical practitioners. The "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve at least 50% of the treated patients exhibiting a reduction in protein aggregation and may also include but is not limited to improved survival rate, more rapid recovery, or improvement or elimination of symptoms and are selected as appropriate measures by those skilled in the art.

[INVENTOR: is this a reasonable definition of "effective amount"? If not, please provide one.]

While it is possible for the expression vector to be administered alone, it is preferable to present it as a pharmaceutical formulation. The formulations of the present invention comprise at least one active ingredient: the expression vector together with one or more pharmaceutically acceptable carriers and optionally other therapeutic ingredients. The carrier(s) must be acceptable in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipients thereof, for example, liposomes. The carriers must also be selected so as not to interfere with the activity of the active ingredient.

The availability of monoclonal antibodies which bind to a specific antigen at distinct and well defined sites has led to a better understanding of the effects of highly specific enzyme-antibody interactions on the enzyme behavior. By appropriate selection it has been possible to isolate those antibodies that are non-inhibitory to biological activity of the enzyme and bind at "strategic locations" on the antigen molecule, resulting in a considerable stabilization effect of the enzyme conformation. Moreover, such monoclonal antibodies, when

properly selected, prove to have a chaperone activity leading to a considerable refolding effect on the enzyme which was already partially heat denatured.

In a model system, renaturation of carboxypeptidase A after heat denaturation in the presence of selected monoclonal antibodies, was followed by recovery of its enzymatic activity. The refolding effect of anti-CPA monoclonal antibodies on heat denatured enzyme depends on the degree of denaturation of the enzyme and on the location of the antigenic site of each antibody. The additivity effect of the pairs of monoclonal antibodies on the refolding process of CPA proved to be dependent on the localization of the antigenic sites of the monoclonal antibodies studied.

Binding of anti-CPA monoclonal antibodies to heat denatured CPA leads to considerable refolding of the enzyme molecule, as judged by the recovery of enzymic activity following immunocomplex formation. The active site of a protein is normally buried inside polypeptide folds. Due to structural fluctuations occurring in the interior of the molecule, however, dynamic channels [Case and Karplus, 1979] may form between the active site

and external regions of the chain [Karplus and Petsko, 1990]. These dynamic fluctuations appear to have the specific function of modulating the reactivity of the protein and allow correlation of the renaturation process with the restored enzymic activity. The epitopes recognized by the mAbs selected for this study (CP 10, CP 9, CP 8) are exposed to the surface solvent and seem to be located in strategic positions in relation to the active site of the enzyme. Binding of CP 9 and CP 8 to the partially unfolded enzyme leads to considerable refolding (20 - 40% of recovered enzymatic activity). The differences in degree of refolding of CPA observed after immunocomplexation with its specific mAbs suggest the existence of special regions on the protein molecule required for folding.

The existence of "sequences kinetically required for folding" was suggested independently [Silen and Agard, 1989] in the case of alphalytic protease and confirmed by *in vitro* denaturation-renaturation experiments. According to these authors, a proregion is required for folding, its omission leading to incorrect folded states. This fragment was assumed to function by direct stabilization of the rate-limiting transition state of the folded intermediates by lowering the

energy barriers along the pathway leading to the native state.

Recognition of the existence of a class of sequences that may play a role in the folding pathway suggests the possibility that such sequences serve not only for stabilization but also may contribute to the efficiency of the folding process. Mabs are able to recognize incompletely folded epitope and induced native conformation in the partially folded protein. The antibody interaction did not affect the structure of the completely unfolded protein.

The above discussion provides a factual basis for the use of monoclonal antibodies and genetically engineered antibody fragments as therapeutics for the prevention of protein aggregation. The methods used with and the utility of the present invention can be shown by the following examples.

EXAMPLES

GENERAL METHODS:

Carboxypeptidase A (CPA)

CPA was obtained as an aqueous crystalline suspension (Sigma Chemical Co., St. Louis, MO). The crystals were washed with double-distilled water, centrifuged, and

Materials

Synthetic peptides Amyloid peptides, Aß 1-40 (Cat. No. A-5813) and Aß 1-28 (Cat. No. A-1084) corresponding to amino acids 1-40 and 1-28 of Aß respectively, were purchased from Sigma Chemical Co., St. Louis, MO, USA).

Amyloid solutions were prepared by dissolving the peptides in water at concentration of 10 mg/ml. The stock solution was stored in aliquotes at -20°C.

Aggregating agents

1. Heparan sulfate (Cat. No. H 5393) was purchased from Sigma Chemical Co., St. Louis, MO, USA).

2. Metal solutions

Stock solutions of metal chlorides were made up from dry salts at concentration of 1mM in TRIS pH 7.4.

Antibodies

Monoclonal antibodies

1. α-Human ß-amyloid 6F/3D was obtained from ACCURATE Chemical and Scientific Corp. (Westbury, NY., USA).

2. Mab AMY 33 was purchased from ZYMED San Francisco, CA, USA.

Polyclonal antibodies

Affinity purified rabbit IgG obtained against the synthetic Alzheimer ß-amyloid (Cat No. 1381431) was purchased from Boehringer- Mannheim, GmbH, Germany.

dissolved in 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5. Insoluble material was removed by centrifugation. The enzyme concentration was derived from the absorbance at 278 nm and was also determined by the Bradford method using bovine serum albumin (BSA) as a standard [Bradford, 1976]. The enzymatic activities of CPA and its immunocomplexes were determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine as peptidase substrate or hippuryl-DL-β-phenyllactic acid as esterase substrate in 0.5 M NaCl/0.05 M Tris-Hcl, pH 7.5, according to Whitaker et al. [1966].

Amyloid

[Source and/or isolation procedure]

Monoclonal Antibody

In general, monoclonal antibodies may be prepared against a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof may be isolated and used as the immunogen. Such proteins or peptides can be used to produce monoclonals by standard production technology well known to those skilled

in the art as further described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and as set forth specifically hereinbelow. The mouse monoclonal antibodies were prepared following the fusion techniques of Kohler and Milstein ~~[1975]~~ (Milstein 1985). Briefly, the technique involves hyperimmunization of an appropriate donor with the protein or peptide fragment, generally a mouse, and isolation of splenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid which has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

The harvested monoclonal antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982. The binding of antibodies to a solid support substrate is also well known in the art. (see for

a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, β-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Purification and characterization of anti-CPA mAbs

The monoclonal antibodies, CP 10, CP 9, CP 8 and CP 32, which interact with CPA at higher binding constant, were chosen for further study. [INVENTOR: why were these monoclonals selected? What criteria are used for selection of mAb that may be chaperones without testing them first for chaperone properties?]

These antibodies were isolated and purified by affinity chromatography on protein A-Sepharose from the corresponding ascites fluids according to Ey et al. [1978]. Protein concentrations were determined according to Bradford [1976] using normal murine IgG as a standard. The preparations of CP 10, CP 8, CP 9

and CP 32 used in this study contained 0.57, 1.30, 2.44 and 1.8 mg protein/ml, respectively.

Purification and characterization of anti-amyloid mAbs

[Source and/or procedure]

Monoclonal antibodies directed against β-amyloid are available from ACCURATE Antibodies and ZYMED. In the present examples monoclonal antibodies _____ were used. [INVENTOR: catalog numbers of any monoclonals that were used]

Protocol for determining effect of monoclonal antibody binding on CPA activity

The enzyme (2 µg in 2 µl of 0.05 M Tris-HCl/0.5 M NaCl buffer, pH 7.5) was incubated for 1 hour at room temperature with increasing amounts of purified mAbs (10-100 µl in the same buffer) and the effect of immuncomplexation on the peptidase and esterase activities was determined spectrophotometrically at 254 nm using either 1 mM hippuryl-L-phenylalanine as peptidase substrate or hippuryl-DL-β-phenyllactic acid as esterase substrate in 0.5 M NaCl/0.05 M Tris-Hcl, pH 7.5, according to Whitaker et al. [1966].

Aggregation assay for amyloid

[procedure]

individual pages 3 and 4

ELISA Test

The antigen-coating solutions (100 µl containing native CPA (10-25µl/ml) in PBS, pH 7.4, were incubated overnight at 4°C in a polystyrene ELISA plate (Costar, Cambridge, MA). Diluted ascites fluid (0.1 ml) containing the desired mAb (1:2000 to 1:18,000 v/v in PBS) was added and incubated at 37°C for 1 hour. The amount of bound mAb was determined with β-galactosidase-linked $F(ab)_2$ fragments of sheep anti-mouse IgG (Amersham International, UK). The apparent binding constant of CPA with its monoclonal antibodies was ~~determined according to the procedure of Pinkard and Weir [1978] and~~ derived from the reciprocal of the free antibody concentration, at which 50% of the maximal binding was achieved.

Additive ELISA.

The assay is based on the method of Friguet et al., [1983]. Saturation concentration

Methods

The aggregation of amyloid was followed by two different sets of experiments:

1. Competitive ELISA assays.

100 ng amyloid was covalently bound on Eupergit-coated ELISA plates by incubation overnight at 4°C. The remaining active epoxy groups on the plate were blocked with non-fat milk. The reaction mixtures containing ß-amyloid (100 ng) and aggregation agents such as heparan sulfate (50 mM) and/or Zn^{2+}, Al^{3+} or Ca^{2+} were incubated for 3h at 37°C. After incubation, the mAbs AMY 33 and 6F/3D were added separately to each reaction mixture (1α) and allowed to interact with the remaining soluble amyloid for another 1 h at 37°C. In parallel, the mAbs were added to the amyloid solutions (100 ng) before exposure at 37°C and incubated together for 3h at 37°C. After incubation the aggregated amyloid preparations were removed by centrifugation at 15,000 rpm for 15' and applied on the ELISA plates previously coated with amyloid. The antibody which did not bind to amyloid in the reaction mixture will bind to the coated amyloid. As shown in Fig. 10, the amount of antibody available for binding the coated antigen will be conversely proportional to the extent of amyloid aggregation. A calibration curve of the amount of antibody bound on the coated amyloid in the absence of soluble added amyloid was performed. The amount of bound antibody was determined using α-mouse antibodies labeled with horse-radish peroxidase (HRP). The enzyme activity of HRP is directly proportional with the amount of monoclonal antibody bound to coated

amyloid. The enzyme activity of horse-radish peroxidase was measured using O-phenylenediamene (OPD) as substrate. The colour developed was measured at OD_{495} using an ELISA reader.

Direct ELISA

The ELISA plates were coated with rabbit polyclonal antibodies raised against ß-amyloid (100 ng/well). The reaction mixtures contained amyloid 10 ng/100 and aggregate reagents, such as heparan sulfate or metal solutions, were exposed to 37°C for 3 h and then incubated for another 1 h at 37°C with mAbs AMY 33 and 6F/3DD at molar ratio 1/1 (300 µg Ab). In another set of experiments the mAbs were added separately to the reaction mixture before incubation at 37°C. After incubation of the reaction mixtures under aforementioned working conditions, 3 h at 37°C, and binding of the mAbs, they were added to ELISA plates coated with polyclonal amyloid antibodies. The amount of soluble amyloid remaining after aggregation was measured as mentioned using second α-mouse antibodies labeled with HRP.

The amount of mAb bound will be proportional to the amount of soluble amyloid remaining after exposure to aggregation conditions, Figs. 11, 12, 13 and 14. The prevention of amyloid aggregation seems to be dependent on the epitope location on he amyloid molecule recognized by a certain antibody.

Aggregation assay for amyloid

[procedure]

ELISA Test

The antigen-coating solutions (100 µl containing native CPA (10-25µl/ml) in PBS, pH 7.4, were incubated overnight at 4°C in a polystyrene ELISA plate (Costar, Cambridge, MA). Diluted ascites fluid (0.1 ml) containing the desired mAb (1:2000 to 1:18,000 v/v in PBS) was added and incubated at 37°C for 1 hour. The amount of bound mAb was determined with β-galactosidase-linked $F(ab)_2$ fragments of sheep anti-mouse IgG (Amersham International, UK). The apparent binding constant of CPA with its monoclonal antibodies was ~~determined according to the procedure of Pinkard and Weir [1978] and~~ derived from the reciprocal of the free antibody concentration, at which 50% of the maximal binding was achieved.

Additive ELISA.

The assay is based on the method of Friguet et al., [1983]. Saturation concentration

curves of adsorbed CPA on ELISA plates (100 µl per well of a solution of 0.05-0.1 µl/ml) with each of the mAbs are determined. For comparison, a double amount of the coated antigen was used as a reference. The amount of bound monoclonal antibodies, alone or in pairs (at the same total concentrations as antibody alone) was determined by using the labeled second antibody, as described previously.

Additivity index (A.I.) was calculated according to the following formula:

$$A.I. = [(2A_{1+2}/A_1+A_2)-1]100$$

A_1, A_2, A_{1+2} represent the adsorptions reached in the ELISA test with the first antibody alone, the second antibody alone and both antibodies together.

[INVENTOR: please provide specific details of how this assay differs from the published literature and are these differences published?]

Determination of effect on refolding of the CPA-monoclonal antibody complexes

CPA (1 mg/ml) in PBS was incubated at 50°C and/or 60°C for various periods of time. The

denaturation of enzyme was followed by determination of the residual enzymic activity. Partially denatured enzyme (2 μ/10 μl of PBS) was incubated with increasing amounts 0.5-2 M/M mAb/CPA (in 100 μl of PBS) of each mAb or with pairs of antibodies (CP 10 + CP 9, CP 10+, CP 8, CP 10 + CP 32). The refolding process of CPA was followed for various periods of time from 10 minutes to 3 hours by determination of recovered enzymatic activity.

[INVENTOR: for the above listed protocols and procedures please indicate what if any changes were made to accommodate amyloid and anti-amyloid monoclonal antibodies. The Patent Office requires that you show that the experiments can be done by having detailed methodology.]

EXAMPLE 1

CHARACTERIZATION OF ANTI-CPA MONOCLONAL ANTIBODIES

In the model system used to test the protocols, the monoclonal chaperone antibodies that were selected by the method of the present invention were shown to bind CPA with relatively

high apparent binding constants (~$10^8 M^{-1}$) and none of them affected either the peptidase or the esterase activities of CPA. Additivity ELISA based on the competition between a pair of mAbs to bind to a constant amount of adsorbed antigen can serve as an indicator of proximity between antigenic sites. The mAbs, either singly or in pairs, were allowed to saturate the adsorbed CPA and the total amount of mAb adsorbed was determined using the labeled second antibody. [INVENTOR: do any of the figures correspond to this example?]

In the presence of the pair (CP 8 and CP 9) (A.I. = 10%), the amount of antibody bound to adsorbed CPA was similar to that found when only a single antibody was employed, suggesting that they bind to the same or close epitope. In the presence of pairs of antibodies, CP 10 and CPA 9 (A.I. = 90%), CP 10 and CP 8 (A.I. = 80%) and CP 10 and CP 32 (A.I. = 70%) the total amount of antibody bound is close to the sum of bound antibody when each mAb is bound separately. Monoclonal antibody CP 10 bind at distinct epitope on CPA molecule relative to that of mAbs CP 9, CP 8 or CP 32.

The antigenic site of CP was identified and behaves like one of the immunodominant

regions of CPA molecules, localized on the surface of the molecule between residues 208-219 [Solomon et al, 1989]. This epitope proved to be especially sensitive to conformational changes in different functional states of the carboxypeptidase A [Solomon et al, 1990]. The other three monoclonal antibodies did not interfere with mAb CP 10 during simultaneous binding to CPA molecule, as suggested by additivity measurements.

EXAMPLE 2

EFFECT ON REFOLDING OF HEAT DENATURED CARBOXYPEPTIDASE A BY MONOCLONAL ANTIBODIES

The unfolding-refolding process of CPA was followed by determination of residual and/or recovered enzymatic activity of the enzyme.

Carboxypeptidase A retained only 10 and 5% of its initial peptidase and esterase activities respectively, after 2 hour incubation at 50°C (in PBS at pH 7.2) (Fig. 1). Incubation at 60°C for 30 minutes leD to total inactivation of the enzyme. As previously shown, immunocomplexation of CPA with each of the mAbs studied, (CP 10, CP 9 and CP 8) at equimolar

ratio followed by incubation at 50°C, resulted in a considerable increase in the thermostability of CPA [Solomon and Balas, 1991].

After denaturation at 50°C, CPA alone was unable to recover its enzymatic activity under the experimental conditions employed.

The unfolding process of CPA was followed, after incubation of the enzyme at 50°C for 30, 60 and 90 minutes. The refolding in the presence of monoclonal antibodies CP 10 showed that the percent recovery of enzymic activity was directly related to the degree of denaturation of CPA (Fig. 2).

Figure 2 is a bar graph of the dependence of refolding of Carboxypeptidase A on the denaturation degree of the enzyme. CPA (1 mg/ml in PBS) was denatured for 30, 60 and 90 min. Aliquotes were withdrawn after each incubation step and reacted with mAb CP 10, at an equimolar ratio, for 1 hour at room temperature. The enzymic activity of the enzyme and its immunocomplexes was determined using esterase substrate. The refolding effect was expressed as percentage of enzymic activity recovered as compared to initial enzymic activity of the enzyme before denaturation. Each experimental

result represents the average of at least three independent measurements.

Incubation of partially heat denatured CPA with other monoclonal antibodies such as CP 9, CP 8 and CP 32 led to different degrees of refolding, as estimated from the recovery of enzymic activity (Fig. 3). Thus, monoclonal antibodies CP 9 and CP 8 cause a 2-3 fold increase in recovered enzyme activity relative to control (CPA alone); monoclonal antibody CP 32 did not induce significant increase in enzymatic activity after immunocomplexation.

Figure 3 is a bar graph of the refolding of Carboxypeptidase A in the presence of the monoclonal antibodies CP 10, CP 9, CP 8 and CP 32. Heat denaturation of CPA was stopped after 60 min and immuncomplexation was allowed to proceed for 1 hour at room temperature. The recovered enzymatic activity was measured as described for Figure 2.

The data obtained show the importance of the location of the antigenic site of each antibody on the refolding process. The refolding process of the enzyme is time-dependent and reaches its maximum after 2 hours at room temperature - the time required for the formation

of the immunocomplex between CPA and its monoclonal antibodies (Fig. 4).

Additivity effects of monoclonal antibody pairs on the refolding process of CPA are shown in Fig. 5. Pairs of antibodies incubated with CPA at equimolar ratio let to increased refolding effect, depending on their antigenic site location. The pair (CP 9 + CP 10), which exhibited the highest stabilization effect against heat denaturation [Solomon and Balas, 1991] also exhibited the highest refolding effect on CPA. The pairs of antibodies CP 10 + CP 8 and CP 10 + CP 32 assisted in the refolding process to a lower extent.

40 ——> Exemple 3

EXAMPLE 4

EFFECT ON REFOLDING OF DENATURED AMYLOID BY MONOCLONAL ANTIBODIES

β-Amyloid (1-40) aggregates in the presence of metal ions (Zn^{2+}, $Al^{??}$) or additional factors such as heparin sulfate. Addition of monoclonal antibody AMY-33 (α-β-amyloid) to the reaction mixture prevents total or partial aggregation of amyloid.

Results

% Aggregation

Fig 2 for 10, 11, 12, 13, 14

please renumber -40- to original page 40

Example 3

CPA aggregate in the presence of Zn^{2+} at 50°C. The immunocomplexation of CPA with some of its selected mAbs restores not only its solubility to the protein but also its enymic activity. See Figs. 7A, 7B, 8 and 9, pages 10 and 11.

Example 4

Prevention of ß-amyloid aggregation by two different monoclonal antibodies.

Amyloid	1.225	100
Amyloid + Antibody	0.6	50
Amyloid + Heparin Sulfate	1.15	93
Amyloid + Heparin Sulfate + Antibody	0.7	60

[INVENTOR: please elaborate on this experiment and the analysis of it. Do you have any additional data?]

Throughout this application various publications are referenced by citation or number. Full citations for the publications referenced by number are listed below. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology which has been used is

intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention may be practiced otherwise than as specifically described.

REFERENCES

[Inventors: Please review the reference list for accuracy and provide us with copies of those references that you have not sent to us previously, designated with a @. We will supply the references indicated with an *.

We noted that you sent to us your working copies of references marked with a #. Some of these references have underlined sections, etc. that are incorporated into your manuscript and disclosure. We will need clean copies to submit to the Patent Office. We can return the working copies to you if you need them.]

@ Amit et al., "Three dimensional structure of an antigen-antibody complex at 2.8A resolution." Nature, 313:156-158 (1985).

@ Blond, S and Goldberg, M., "Partly native epitopes are already present on early intermediates in the folding of tryptophan synthase." Proc. Natl. Acad. Sci., 84:1147-1151 (1987).

@ Bradford, M.M., "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal. Biochem., 72:248-254 (1976).

Carlson, J.D. and Yarmush, M.L., "Antibody assisted protein refolding." Bio/Technol., 10:86-91 (1992).

@ Case, D.A. and Karplus, M., "Dynamic of ligand binding to home proteins." J. Molec. Biol., 132:343-368 (1979).

@ De Young et al., "Aggregation of Globular Proteins", Accounts of Chemical Research, 26:614-?? (1993). [We need complete article]

Dueñas et al., "Intra- and Extracellular Expression of an scFv Antibody Fragment in *E. coli*: Effect of Bacterial Strains and Pathway Engineering Using GroES/L Chaperonins" BioTechniques, 16:476-483 (1994).

@ Edwards, B.J. and Morley, J.E., "Amylin", Life Sci., (1992)

Ellis et al., "The Molecular Chaperone Concept", Biochem. Soc. Symp., 55:145-153 (1989).

@ Ey et al., "Isolation of pure IgG1, IgG2a and IgG2b immunoglobulins from mouse serum using Protein A-Sepharose." Immunochemistry, 15:429-436 (1978).

@ Frauenfelder et al., "Temperature dependent X-ray diffraction as a probe of protein structural dynamics." Nature, 280:558-563 (1979).

@ Friguet et al., "A convenient enzyme-linked immunosorbent assay or testing whether monoclonal antibodies recognize the same antigenic site. Application of hybridoma specific for the B_2-subunit of *E. coli* tryptophan-synthase." J. Immunol. Meth., 60:351-358 (1985).

Goloubinoff et al., "Reconstitution of active dimeric ribulose bisphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP" Nature, 342:884-889 (1989).

Haber, E., "Engineered Antibodies as Pharmacological Tools", Immunological Reviews, 130:189-212 (1992).

@ Jaenicke, R., "Protein Folding: Local structures, domains, subunits and assemblies." Biochemistry, 30:3147-3161 (1991).

@ Karplus, M. and Petsko, G.A., "Molecular dynamics simulations in biology." Nature, 347, pp. 631-639 (1990).

@ Kastin, et al, "Evidence for peptide aggregation", Pharmacol Biochem Behav 21:969-973 (1984).

@ Kohler, G. and Milstein, C., "Continuous cultures of fused cells secreting antibody of predefined specificity." Nature, 256, pp. 495-497 (1975).

Marasco et al., "Design, intracellular expression, and activity of a human anti-human immunodeficiency virus type 1 gp120 single-chain antibody", Proc. Natl. Acad. Sci. USA, 90:7889-7893 (August 1993).

Mendoza et al., "Chaperonin cpn60 from *Escherichia coli* Protects the Mitochondrial Enzyme Rhodanese against Heat Inactivation and Supports Folding as

Elevated Temperatures", J. Biolog. Chem., 267(25):17631-17634 (1992).

@ Oakley et al, "Electron microscopic appearances and implications of neuropeptide fibrillary forms", Neuropeptides 2:1-11 (1981).

@ Pelham, H.R.B., "Speculations on the function of the major heat shock and glucose regulated proteins", Cells, 46:959-961 (1986).

@ Pinkard, R.N. and Weir, D.M., in Handbook of Exp. Immunology (Weir, D.M. ed.). Chapter 16, pp. 16.1-16.20, Blackwell Scientific, Oxford (1978).

@ Rothman, J.E., "Polypeptide chain binding proteins: catalysis of protein folding and related processes in cells." Cell, 59:591-601 (1989).

@ Selkoe, D.J., "The molecular pathology of Alzheimer's Disease" Neuron, 6:487-498 (1991).

@ Silen, J.L. and Agard, D.A., "The x-lytic protease pro-region does not require a physical linkage to activate the protease domain in vivo." Nature, 341:462-464 (1989).

@ Skowyra et al., "The *E. coli* dnaK gene product, the hsp70 homolog, can reactivate heat-inactivated RNA polymerase in an ATP hydrolysis-dependent manner." Cell, 62:939-944 (1990).

Solomon et al., "Localization of a highly immunogenic region of Carboxypeptidase A recognized by three different monoclonal antibodies and their use in the detection of subtle conformational alterations in this enzyme region." Biochemistry, 28:1235-1241 (1989).

@ Solomon et al., "Catalytic and conformational changes induced by limited subtilisin cleavage of bovine Carboxypeptidase A." Biochemistry, 29:7303-7309 (1990).

Solomon, B. and Balas, N., "Thermostabilization of Carboxypeptidase A by interaction with its monoclonal antibodies." Biotechnol. Appl. Biochem., 14:202-211 (1991).

@ Solomon, B. and ~~Balas, N.~~, "Chaperone-like effect of monoclonal antibodies on refolding of heat denatured Carboxypeptidase A.J. Molec. Recog." (in press) (1994).

Travis, J. "Putting Antibodies to Work Inside Cells", Science, Vol. 261:1114 (27 August 1993).

@ Vallee, B.L. and Riordan, J.F., "Chemical approaches to the properties of active site of enzymes." Ann.Rev.Biochem., 38:733-794. (1969).

Vandenbroeck et al., "Refolding and single-step purification of porcine interferon-γ from *Escherichia coli* inclusion bodies - Conditions for reconstitution of dimeric IFN-γ", Eur. J. Biochem., 215:481-486 (1993).

* Welch, W.J., "How Cells Respond to Stress", Scientific American, pp. 56-64 (May 1993).

Wetzel, R. "Mutations and off-pathway aggregation of proteins", Tibtech, 12:193-198 (May 1994).

@ Whitaker et al., "The kinetics of some Carboxypeptidase A and acetylcarboxypeptidase A catalyzed hydrolysis." Biochemistry, 5:386-392 (1966).

CLAIMS

What is claimed is:

1. A method of selecting monoclonal chaperone antibodies that have characteristics including binding to a target molecule epitope with a high binding constant and are non-inhibitory to the biological activity of the target molecule including the steps of

denaturing the target molecule,

mixing the denatured target molecule with the chaperone monoclonal antibody,

renaturing the mixture to produce nonaggregated target molecule,

testing the renatured nonaggregated target molecule coupled to the monoclonal chaperone molecule for bioactivity.

2. The method of claim 1 further characterized by the target molecule being β-amyloid.

3. The method of treating a protein aggregation disease including the steps of

preparing at least human monoclonal antibody that binds to an aggregating protein which is the cause of a disease and which prevents aggregation while allowing bioactivity;

creating an expression vector comprising nucleic acid including a sequence which encodes in expressible form the human monoclonal antibody that binds to an aggregating protein and which prevents aggregation; and

administering the expression vector.

4. The method of claim 3 wherein the human monoclonal antibody that binds to an aggregating protein and which prevents aggregation while allowing bioactivity is an anti-β-amyloid.

[INVENTOR: have you produced this monoclonal antibody or have you only used commercial sources?]

5. The method of claim 3 wherein the human monoclonal antibody that binds to an aggregating protein and which prevents

aggregation while allowing bioactivity is a single chain monoclonal antibody.

6. The method of claim 3 wherein at least two human monoclonal antibodies are used.

7. The method of claim 3 further characterized by delivering the expression vector to the patient.

8. The method of claim 3 wherein the expression vector is a virus.

9. The method of claim 8 wherein the nucleic acid is RNA.

10. The method of claim 9 wherein the expression vector is a disable retro virus.

11. The method of claim 9 wherein the expression vector is a retroviral shuttle vector

12. The method of claim 8 wherein the expression vector is vaccinia virus.

13. The method of claim 8 wherein the expression vector is an adenovirus.

14. The method of claim 3 wherein the expression vector is a plasmid.

15. A pharmaceutical composition comprising the expression vector as set forth in claim 3 and a pharmaceutically acceptable carrier.

16. An expression vector for use as a therapeutic agent which comprises nucleic acid including at least one sequence which encodes in expressible form a human monoclonal antibody which a human monoclonal antibody that binds to an aggregating protein which is the cause of a disease and which prevents aggregation.

17. The expression vector as set forth in claim 16 wherein the human monoclonal antibody is an anti-β-amyloid monoclonal antibody.

18. The expression vector as set forth in claim 16 wherein the human monoclonal antibody is a single chain monoclonal antibody.

Monda October 03, 1994
Form Doc2STR#251:30

#251:1
chP-306/Your Ref: 1180

Message

om: Jackie Walton
ubject: P-306/Your Ref: 1180
o: Hananel Kvatinsky
c:
ttachments: TAV-P306.APP 71K

Attached is a draft patent application for your review and the review and comment by the inventor. We are sending, via DHL courier, copies of the PCT patents for the inventors review. We have removed the Sequence Listings from each patent to reduce costs. Also, enclosed is an Information Sheet for the preparation of formal papers.

EXHIBIT A

TEL AVIV UNIVERSITY אוניברסיטת תל-אביב

GEORGE S. WISE FACULTY OF LIFE SCIENCES
DEPT. OF MOLECULAR MICROBIOLOGY & BIOTECHNOLOGY

הפקולטה למדעי החיים ע"ש ג'ורג' ס. וייז
המחלקה למיקרוביולוגיה מולקולרית וביוטכנולוגיה

2nd November 1994

Dr. Kenneth I. Kohn
Reising, Ethington, Barnard
Perry & Milton
Columbia Center
201 W. Big Weaver Suite 400
P.O.Box 4390
Troy, Michigan 48099
USA

Dear Dr. Kohn,

As agreed during your visit here I am sending you herewith the modifications and supplementary data regarding the patent.

Please contact me directly if you need any assistence. My telephone number is (972-3) 6409711, and fax number (972-3) 6409407.

I am now waiting for your agreement to go ahead and publish the papers and make the necessary application for financial assistance to continue the project.

I do hope you enjoyed your stay in Israel and thank you for all your cooperation.

With best wishes.

Sincerely yours,

Beka Solomon

Beka Solomon, Ph.D.

EXHIBIT B

קרית האוניברסיטה, רמת-אביב, תל-אביב 69978; טל' 03-6408111; פקס' 03-6409407
RAMAT AVIV, 69978 TEL AVIV, ISRAEL; TEL. 03-6408111, FAX: 972-3-6409407

From Beka Solomon :

1. Claim 1 : Please change only lines 11, 12 as follows:
   mixing of target proteings with monoclonal antibodies,
   exposure to denaturation...

2. The antibody building site of the mopnoclonal abtibody =
   = peptyde which mimics bunding site of the abtibody to the antigene.

From me:

No need to send us the revised draft before filing if you are sure that it is ready to be filed.

regards

Hananel

EXHIBIT C

STR#251:1 Friday, November 11, 1994 07:38:39 PM
Search**Beka Solomon** Form Doc2STR#251:30

Friday, November 11, 1994 10:10:16 AM
Message

From: Ken Kohn
Subject: Beka Solomon
To: Hananel Kvatinsky
Cc: Jackie Walton

Howdy. How is everything in the Holyland?
While reviewing all of the papers that I recieved while I was in Israel, I noted that I made a mistake. It turns out that the papers that I recieved at the hotel were from Wientraub, and not Dr. Solomon. My apologies for my oversight. It must have been jetlag. Accordingly, I still need to recieve the materials that I reqested from Beka. I can then finalize and file her application.
Again, I apologize for any inconvevience this may have caused.
KIK

EXHIBIT D

[redacted] Friday, November 11, 1994 10:10:16 AM
Message

From: Ken Kohn
Subject: Beka Solomon
To: Hananel Kvatinsky
Cc: Jackie Walton

Howdy. How is everything in the Holyland?
While reviewing all of the papers that I recieved while I was in Israel, I noted that I made a mistake. It turns out that the papers that I recieved at the hotel were from Wientraub, and not Dr. Solomon. My apologies for my oversight. It must have been jetlag. Accordingly, I still need to recieve the materials that I reqested from Beka. I can then finalize and file her application.
Again, I apologize for any inconvevience this may have caused.
KIK

EXHIBIT E

Monday, November 14, 1994 09:12:38 AM
Message

From: Ken Kohn
Hananel Kvatinsky

Subject: Fwd: courier

To: Jackie Walton

Shalom

This is just to inform you that we have shipped Bekka Solomon's material by Courier Network (in NY 212-675-6876, 800-222-9951) . Their airbill No. is 267214. Courier Network are shipping via DHL in the US>

Let me know if the package does not arrive until Wednesday.

Hananel

ps : The confirmation copy regarding the Boxman patent is also there.

HK

EXHIBIT F

R A M O T OF TEL AVIV UNIVERSITY

ר מ ו ת ע"י אוניברסיטת תל אביב

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ת.ד. 39296
תל אביב 61392
טל. 03-6428765

FAX MESSAGE

DATE: 16-NOV-94 תאריך: No. of pages 1+10 דפים:

TO: Dr. Kenneth Kohn אל: OUR REF: (1180) מספרנו:

Company: REISING ETHINGTON חברת: FROM: Hanan el Kvatinsky השולח:

Fax: 00-1-810-687-4071 פקס: Fax: (972)-3-6429865 פקס:

Your Ref: P-306

MESSAGE: תודעה:

Dear Ken:

I am enclosing an article which Dr. Becca Solomon wants to send to Nature.

Please go over it and let us know if, in your opinion, there is something you would not disclose even after a patent application has been filed.

In addition, since the article might be sent before the patent application is filed let us know if you think there is any problem with that.

Regards

Hananel Kvatinsky
Assist R&D Manager

EXHIBIT G

[redacted] Thursday, November 17, 1994 10:24:34 AM
Message

From: Ken Kohn
Hananel Kvatinsky

Subject: Fwd: ??

To: Jackie Walton

Shalom

I want to verify that you have revceived my last messages and material DHLed to you.

This goes to Boxamn's patent, Beka Solomon's patent (and article) and Wientroub/Bnayahu proposed patent applications

regards

Hananel

EXHIBIT H

EXHIBIT I

REORDER 805235698-2 QTY 250

DHL WORLDWIDE EXPRESS

Shipment Airwaybill
(Non negotiable)
1-800-CALL-DHL in USA only

PPF CMG 7 6 3 1 2 8 5 9 6 6

Quote this shipment number in an inquiry

PP

11/16/94 1

ORIGIN: DTX DESTINATION:

DHL AIRWAYS, INC.® • 333 TWIN DOLPHIN DRIVE, REDWOOD CITY, CA 94065 **Original (DHL Billing) Copy**

Complete sections 1-5. You are making 5 copies, please type

1 From (Shipper)

Account no.: 805235698

Shipper's reference: #2290 P-306 (TelAviv)

Company name: REISING ETHINGTON BARNARD

Shipper's name: Sue Trudel/Kenneth I. Kohn

Address:
SUITE 400
201 W BIG BEAVER RD
TROY MI

Zip code (required): 480844152

Phone/Fax/Telex circle one: (313)689-3500

2 To (Recipient)

Company name: RAMOT of Tel Aviv University

Attention: Mr. Hananel Kvatinsky

Delivery address *DHL cannot deliver to a PO Box*

32, Haim Levanon Street
Tel Aviv 61392 ISRAEL

Zip/Postcode (required): 61392

Phone/Fax/Telex circle one: 011-972-3-6408113

5 Shipper's authorization and signature

I/we agree that DHL's standard terms apply to this shipment and limit DHL's liability for loss or damage to US $ 100. The Warsaw Convention may also apply (see reverse). I/we authorize DHL to complete other documents necessary to export this shipment. I/we understand that insurance is available on request for an extra charge. I/we agree to pay all charges if the recipient or third party does not pay. I/we understand that DHL DOES NOT TRANSPORT CASH.

Signature: S. Trudel

Date: 12/16/94

REV 2/94

3 Shipment details

Services

- [ ] U.S. DOMESTIC
- [x] INTERNATIONAL DOCUMENT
- [ ] INTERNATIONAL NON DOCUMENT
- [ ] WORLDMAIL 1st / APM / 2nd *circle one*

Special Services *extra charges may apply*

- [ ] SATURDAY DELIVERY
- [ ] POD ____
- [ ] OTHER ____ *specify*

Payment Options *not all options available to all countries*

- [x] Shipper's account
- [x] Recipient
- [ ] Third party

Acct. No. ____

- [ ] Cash/Check/Credit Card *circle one*

No. ____

Expires ____ Type ____

Shipment insurance *if desired, enter amount below*

U.S. $ ____ *Insured value*

Full description of contents

Business documents

International non document shipments only
Attach original and three copies of a Commercial Invoice

Declared value for customs (in US $): ndv

Export license no./Symbol *if applicable*

Harmonized Sched. B no. *if applic.*

Ultimate destination

Shipper's EIN/SSN

This shipment is licensed by the US for the ultimate destination named above. Diversion contrary to US Law is prohibited.

Destination duties/taxes *If left blank recipient pays duties/taxes*

- [ ] Recipient
- [ ] Shipper
- [ ] Other ____

4 Pcs/Weight/Size

No. of pieces

Weight *If DHL Express Document packaging is used, enter XD* lb

Dimensions *in inches* length x width x height

DIMENSIONAL/CHARGED WEIGHT lb

CODES	CHARGES
	Services
	Special services
	Insurance
Drop Box/ Exp. Center	
TOTAL	

TRANSPORT COLLECT STICKER No.

PICKED UP BY

Time

Date

1A

1B-2

Ultra-Image® MCP® PATENTED 2010

Message

rom: Jackie Walton
ubject: P-306/Your Ref: 1180
o: Hananel Kvatinsky
c:
ttachments: TAV-P306.APP 71K

1180

CONFIRMATION COPY

ttached is a draft patent application for your review and the review and comment by the nventor. We are sending, via DHL courier, copies of the PCT patents for the inventors eview. We have removed the Sequence Listings from each patent to reduce costs. lso, enclosed is an Information Sheet for the preparation of formal papers.

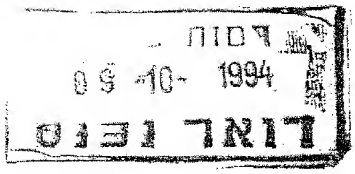

EXHIBIT J

Wednesday, November 02, 1994 09:02:50 AM
Message

From: Ken Kohn
Subject: Deliveries
To: Hananel Kvatinsky

Hi. We're getting ready to go and I hae recieved the package from Beka Solomon but not from Dr W. If it arrives before I leave, I'll let you know. Otherwise, I'll look forward to recieving it by mail. It was a great stay and I look forward to seeing you in January or early March. Be well.

178

EXHIBIT L

Friday, November 11, 1994 10:10:16 AM
Message

From: Ken Kohn
Subject: Beka Solomon
To: Hananel Kvatinsky
Cc: Jackie Walton

Howdy. How is everything in the Holyland?
While reviewing all of the papers that I recieved while I was in Israel, I noted that I made a mistake. It turns out that the papers that I recieved at the hotel were from Wientraub, and not Dr. Solomon. My apologies for my oversight. It must have been jetlag. Accordingly, I still need to recieve the materials that I reqested from Beka. I can then finalize and file her application.
Again, I apologize for any inconvevience this may have caused.
KIK

1180

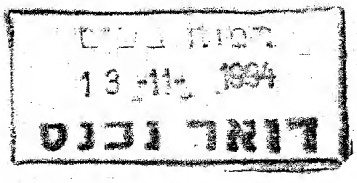

EXHIBIT M

R A M O T OF TEL-AVIV UNIVERSITY

ר מ ו ת ע"י אוניברסיטת תל אביב

32 Haim Levanon St.
P.O.Box 39296
Tel-Aviv 61392
Phone: (972)-3-6428765
Internet: ramot@ccsg.tau.ac.il

רח' חיים לבנון 32
ת.ד. 39296
תל אביב 61392
טל. 03-6428765

FAX MESSAGE

DATE: 16-NOV-94 :תאריך

No. of pages 1+10 :מס'

TO: Dr. Kenneth Kohn :אל

Company: REISING ETHINGTON :חברה

Fax: 00-1-810-689-4071 :פקס

OUR REF: (1180) :מספרנו

FROM: Hanan el Kvatinsky :השולח

Fax: (972)-3-6429865 :פקס

Your Ref: P-306

MESSAGE: :הודעה

Dear Ken:

I am enclosing an article which Dr. Bekka Solomon wants to send to Nature.

Please go over it and let us know if, in your opinion, there is something you would not disclose even after a patent application has been filed.

In addition, since the article might be sent before the patent application is filed let us know if you think there is any problem with that.

Regards.

Hananel Kvatinsky
Ass'st RSO Manager.

EXHIBIT N

INHIBITION OF β-AMYLOID AGGREGATION BY MONOCLONAL ANTIBODIES

Beka Solomon, Rela Koppel and Tamar Katzav
Department of Molecular Microbiology and Biotechnology
Tel-Aviv University, Ramat Aviv, Israel

Evidence that β-amyloid - the hallmark of Alzheimer's disease (1,2) - has both neurite promoting and neurotoxic properties, and that the expression of these opposing effects is related to peptide aggregation (3,4,5), focuses the development of appropriate approaches toward reducing or eliminating the extent of amyloid deposition (6,7). In the present study, the aggregation process of synthetic β-amyloid was followed using its immunocomplexation with two specific monoclonal antibodies: AMY-33 and 6F/3D, raised against β-amyloid fragments comprising the amino acids 1-28 and 8-17, respectively. β-Amyloid aggregation was induced by incubation for 3 h at 37°C in the presence of heparan sulfate (8) and metal ions (9-11). We found that the addition of antibodies to the β-Amyloid preparations before exposure to the abovementioned experimental conditions considerably inhibits its aggregation. This effect of monoclonal antibodies in the prevention of β-amyloid aggregation was found to be related to the localization of the antigen sites and to the nature of the aggregating agents. This study provides a factual basis for the use of monoclonal antibodies as therapeutic approaches for the prevention of *in vivo* β-amyloid aggregation, associated with Alzheimer's disease.

Under physiological conditions, the synthetic β-amyloid peptide (βA4) adopts an aggregated form and shows a change in its biological effects on hippocampal neurons from neurite promoting to

EXHIBIT O

neurotoxicity (3,12). The so-called pathological chaperones (13), and certain metals (9-11) proposed as risk factors in Alzheimer's disease, favour the β-amyloid cascade aggregation. The insoluble amyloid stability was one of the more unsurmountable problems in the initial characterization of the constituent proteins of the isolated plaque cores from brains affected by Alzheimer's disease. Strongly denaturing conditions, such as high concentrations of urea, guanidine-HCl or extreme pH, are required to break and dissolve such aggregation (2,14).

Approaches have recently been focused on the development of potent and selective inhibitors toward reducing or eliminating the extent of amyloid deposition, and on the development of anti-pathological chaperones (6,7,13).

The availability of monoclonal antibodies (mAbs) which bind to a specific antigen at distinct and well-defined sites enabled a better understanding of the effect of highly specific antigen-antibody interaction on the antigen behaviour. Complementary conformation between the interacting regions of the antibody with its antigen confers high specificity and stability to the immunocomplex (15). Monoclonal antibodies produced against a specific antigen toward a well-defined antigenic site were found to exhibit a selective folding effect of the respective antigen (15-17). By appropriate selection, it has been possible to isolate monoclonal antibodies that are non-inhibitory to the biological activity of the antigen and which bind to strategic locations on the protein or peptide molecule. These antibodies proved to have

a chaperone-like activity, leading to considerable refolding effect of the partially denatured antigen by recognizing incompletely folded epitopes and inducing native conformation (manuscript submitted). Such epitopes suggest the existence of a class of sequences in the protein molecule that may play a role in the folding-unfolding or solubilization-aggregation pathways (18,19). The data available in literature suggest that for practically all the antigens it might be possible to prepare monoclonal antibodies which bind to preselected epitopes with a high affinity to the antigen without affecting their catalytic activity.

Insert A

As described below, we investigated the immunocomplexation effect on the *in vitro* aggregation of β-amyloid. Aggregation of β-amyloid was found to be dependent on the pH, peptide concentration, temperature and time of incubation (20). In our experiments, the aggregation of β-amyloid was performed by incubation of aqueous solution of BA4 (10 mg/ml) for 3 h at 37°C. The β-amyloid aggregation was followed by ELISA measurements using two different commercially available monoclonal antibodies raised against β-amyloid: α-human β-amyloid 6F/3D obtained from Accurate Chemical and Scientific Corp, Westbury, N.J. USA, and mAb AMY 33 (21), purchased from Zymed, San Francisco, CA, USA, raised against peptides 8-17 and 1-28, respectively, of the β-amyloid. The addition of the antibodies was made before or after exposure of synthetic β-amyloid to the aggregation process (Fig. 1A,B). The aggregation of the β-amyloid was performed in the presence of heparan sulfate and/or metal ions, such as Zn^{2+} and Al^{3+}. The

antibody AMY-33, which is supposed to recognize an epitope spanned between the sequence 1-28, inhibits the β-amyloid aggregation occurring in the presence or absence of heparan sulfate (Fig. 1A). Any significant effect on metal-induced amyloid aggregation was observed under the same experimental conditions. The mAb 6F/3D, recognizing an epitope located between the sequence 8-17 of the β-amyloid, interfers with Zn^{2+}-induced aggregation, suggesting the partial solubilization effect of already aggregated β-amyloid, but has no effect on other aggregating agents (Fig. 1B).

Metals, such as Zn^{2+} and Al^{3+}, have been proposed as risk factors for Alzheimer's disease development (9-11). The aggregation of βA4 induced by aluminium is distinguishable from that induced by Zn in terms of rate, extent, pH and temperature dependence (9). Although the precise site of interaction of metal ions and βA4 is not clarified, several residues in βA4 are candidates for metal binding. The βA4 histidine residues (His_{13}-His_{14}) may be implicated in fibril formation and it is conceivable that at least H_{13} remains available for intermolecular electrostatic interactions between anti-parallel chains (22). The site defined by Val_{12}-His_{13}-His_{14}-Glu_{15}-Lys_{16}-Leu_{17} has been identified as a sequence contained heparan sulfate binding domain (8) and His_{13} and Lys_{16} are supposed to provide the cationic binding sites being exposed on the same face of the peptide β sheet (22). Binding of mAb AMY-33 to βA4 prevents self-aggregation of the β-amyloid, probably by recognizing the sequence 26-28 located in the proposed aggregation fragment comprising the amino acids between 25-35 (23) (Fig. 2).

This antibody prevents intramolecular aggregation occurring in the presence of heparan sulfate, which is supposed to affect only the aggregation of preexisting amyloid fibers (8). Inhibition of ß-amyloid aggregation in the presence of mAb 6F/3D was partially effective only in the presence of Zn^{2+}. Search for the appropriate monoclonal antibodies against ß-amyloid directed toward epitopes supposed to be involved in metal or other aggregation factors, without interfering with the soluble ß-amyloid functions, is being intensively carried out.

On the basis of our findings regarding other antigen-antibody systems studied (24,25), the formation of the immunocomplexes with selected, highly specific monoclonal antibodies, should provide a general and convenient method to prevent aggregation of the proteins without affecting their biological properties.

At least 15 different polypeptides are known to be capable of causing *in vivo* different forms of amyloidosis via their deposition in particular organs or tissues as insoluble protein fibrils.

Recent advances in antibody engineering technology, as well as in the development of suitable delivery systems (26-29) may make it possible to develop functional small antibody fragments to serve as therapeutic chaperones for the treatment of Alzheimer's disease as well as other human amyloidosis diseases by gene based therapies.

Fig. 1. Aggregation of β-amyloid (1-40) in the absence (-) and in the presence (+) of monoclonal antibodies AMY-33 (A) 6F/3D (B) followed by ELISA. 1. β-amyloid alone; 2. β-amyloid + 50 mM heparan sulfate; 3. β-amyloid + 10^{-6}M $AlCl_3$; 4. β-amyloid + 10^{-5} M $ZnCl_2$.

Methods

The ELISA plates were coated with rabbit polyclonal antibodies (Boeringer-Mannheim) raised against synthetic β-amyloid (1-40) (Sigma) (100 ng/well) via covalent attachment to epoxy-coated ELISA plates by incubation at 4°C for 16 h. The residual epoxy groups were blocked by non-fat milk. The reaction mixtures containing aqueous solution of β-amyloid (100 ng/ml), heparan sulfate (50 mM) and/or chloride metal solutions (10^{-3}M at pH 6.5), were exposed to 37°C for 2 h. The aggregated β-amyloid preparations were removed by centrifugation at 15,000 g for 15'. The residual soluble β-amyloid was incubated for another 1 h at 37°C with mAbs AMY 33 and/or 6F/3D at equal molar ratio antibody/antigen. In another set of experiments the mAbs were added to the reaction mixtures before incubation at 37°C and then incubated together for 2 h at 37°C. After the incubation period the immunocomplexed amyloid preparations were added to the ELISA plates, previously coated with polyclonal anti-amyloid antibodies. The amount of mAb bound will be proportional to the amount of soluble amyloid which remained after exposure to aggregation conditions.

The amount of bound antibody was determined using α-mouse second antibodies labeled with horseradish peroxidase (HRP). The

enzyme activity of HRP is directly proportional with the amount of residual amyloid bound to rabbit polyclonal antibodies. The enzyme activity of HRP was measured using O-phenylenediamane (OPD) as substrate. The colour developed was measured at A_{492} using an ELISA reader. Data represent the mean of triplicate determinations. The standard deviation of the intra-assay and interassays were less than 5% in all cases.

Fig. 2. Schematic diagramms of ß-amyloid (1-40). Horizontal lines represent the regions against which monoclonal antibodies were produced. Vertical lines and rectangular hatches represent the heparan sulfate binding sites (residues 12-17), the proposed toxic fragment (residues 25-35) and the putative epitope of mAb AMY-33 (sequence 25-28). The detailed sequences of the heparan sulfate site and putative epitope of mAb AMY-33 are also shown.

References

1. Selkoe, D.J. Neuron 6, 487-498, (1991).
2. Fraser, P.E, Levesque, L. and McLachlan, D.R. Clin. Biochem. 26, 339-349, (1993).
3. Pike, C.J., Walencewicz, A.J., Glabe, C.G. and Cotman, C.W. Brain Research. 563, 311-314, (1991).
4. Koo, E. H., Park, L. and Selkoe, D.J. Proc. natn. Acad.Sci. USA. 90, 4748-4752, (1993).
5. Maruyama, K. et al. Gerontology 40(suppl2), 57-64, (1994).
6. Snyder, S.W. et al. Biophys. J. 67, 1216-1229, (1994).
7. Schwarzman, A.L. et al. Proc. natn. Acad. Sci. USA., 91, 8368-8372 (1994).
8. Fraser, P.E., Nguyen, J.T., Chin, D.T. and Kirschner, D.A. J. Neurochem., 59, 1531-1540 (1992).
9. Mantyh, P.W. et al. J. Neurochem. 61, 1171-1173 (1993).
10. Frederickson, C.J. Int. Rev. Neurobiol. 31, 145-238 (1989).
11. McLachlan, D.R.C. et al. Lancet, 337, 1304-1308 (1991).
12. Pike, C.J. et al. J. Neurosci. 13, 1676-1681 (1993).
13. Wisniewski, T. et al. Biochem. Biophys. Res. Commun., 192, 359-365 (1993).
14. Masters, C.L. et al. Proc. natn. Acad. Sci. USA. 82, 4245-4249 (1985).
15. Goldberg, M.E. Trends Biochem., 16, 358-362 (1991).
16. Blond, S. and Goldberg, M. Proc. natl. Acad. Sci. USA 84, 1147-1151 (1987).

17. Carlson, J.D. and Yarmush, M.L. *Biotechnol.* 10, 86-91 (1992).

18. Karplus, M. and Petsko, G.A. *Nature*, 347, 631-639 (1990).

19. Silen, J.L. and Agard, D.A. *Nature*, 341, 462-464 (1989).

20. Murdick, D. et al. *J. Biol. Chem.* 267, 546-554 (1992).

21. Stern, R.A., Troyanowski J.Q. and Lee, V.M.Y. *FEBS Lett.*, 264, 43-47 (1990).

22. Telefous, J., Marcinowsky, K.J., Klopman, G. and Zagorski, M.G. *Biochemistry*, 33, 7788-7795 (1994).

23. Yankner, B.A., Duffy, L.K. and Kirschner, D.A. *Science*, 250, 279-282 (1990).

24. Solomon, B. et al. *Biochemistry* 28, 1235-1241 (1989).

25. Solomon, B. and Balas, N. *Biotech. Appl. Biochem.* 14, 202-211 (1991).

26. Haber, E. *Immunolog. Reviews*, 130, 189-212 (1992).

27. Pluckthun, A. *Immunolog. Reviews* 130, 151-188 (1992).

28. Travis, J. *Science*, 261, 1114 (1993).

29. Marasco, W.A., Haseltine, W.A. and Chen, S. *Proc. natl. Acad. Sci. USA* 90, 7889-7893 (1993).

Acknowledgments: We thank Professor Ephraim Katchalski-Katzir for critical evaluation of the manuscript, to Yael Dror for graphics and to Faybie Margolin for preparation of the manuscript.

Fig. 1.

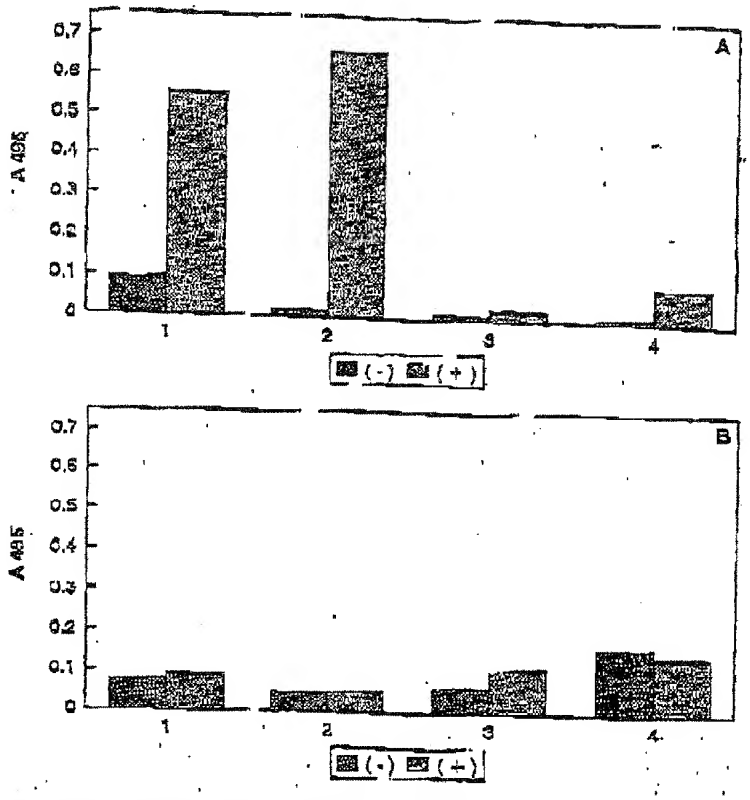
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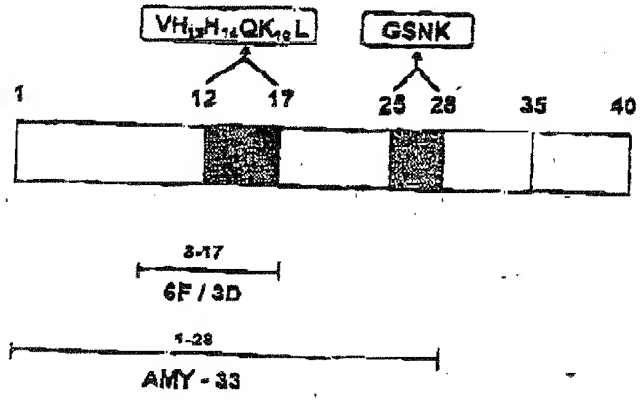
Fig 2.


VH12H14QK16L
GSNK
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17
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35
40
3-17
6F / 3D
1-28
AMY - 33

Friday, December 02, 1994 08:48:31 AM
Message

From: Ken Kohn
Subject: Applications
To: Hananel Kvatinsky
Cc: Jackie Walton

You should recieve a final draft of Beka Solomon by Sunday or Monday. There were so many comment and additions (some in Hebrew), that I would like her to review it. Tell her not to worry re the publication because if she reviews it quickly, we can then file it immediately. There should be little if no further changes by her on this draft.
We recieved Wietraub's stuff and will proceed immediately with the preparation of a pat app.
I hope that you had a nice weekend.
KIK

1180

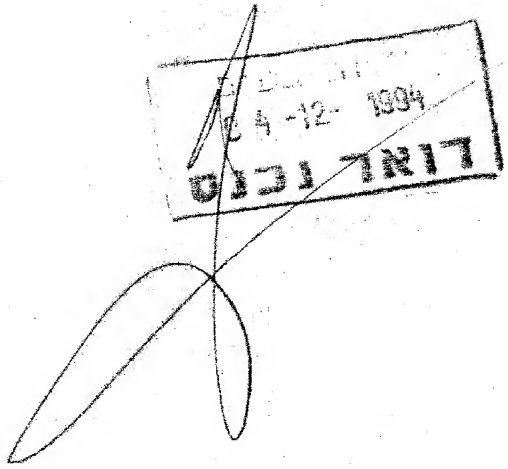

EXHIBIT P

Friday, December 02, 1994 04:30:58 PM
Message

From: Jackie Walton
Subject: Beka Solomon Appln. P-306
To: Hananel Kvatinsky
Attachments: FORM7-3 8K

Hi Hananel:

So that we can prepare the formal papers for the inventors to execute we will need the full name, address and citizenship of each inventor in the order they should appear on the patent. Is the Assignment to be the normal one.

Please find attached a Verified Statement for execution and Faxing back to us.

If You have any questions, please contact me.

Thanks
Jackie

1/80

EXHIBIT Q

Monday, December 05, 1994 08:35:37 AM
Message

From: Jackie Walton
Subject: Tel-Aviv
To: Hananel Kvatinsky
Attachments:

TAV-P306.7-4	8K
TAV-P306.ASN	4K
TAV-P306.DEC	34K

Hi Hananel:

Sorry for the confusion. Please find attached the various documents for execution.

Jackie

EXHIBIT R

R|A|M|O|T OF TEL AVIV UNIVERSITY
רמות
UNIVERSITY AUTHORITY FOR APPLIED RESEARCH AND INDUSTRIAL DEVELOPMENT LTD.

December 7, 1994
Our Ref: (1180/1)-1079
Your Ref: P-306

Dr. Kenneth I. Kohn
Reising, Ethington, Barnard, Perry & Milton
and
Learman & McCulloch
P.O.Box 4390
Troy, Michigan 48099
U.S.A.

FAX: 00-1-(313) 689-4071
Confirmation by courier

Dear Ken:

RE: Prevention of Aggregation . Inventor: Beka Solomon

I am enclosing, to the confirmation of this letter:

A. A letter from Dr. Beka Solomon with her remarks and changer to the patent application.

B. The references you have requested from Dr. Solomon.

C. The power of attorney and assignment signed by the inventor.

D. Small Business Declaration signed by RAMOT.

Regards,

Hananel Kvatinsky
Assistant R & D Manager
Patents & Technology Transfer

cc: (w/o encl.) Dr. Beka Solomon

EXHIBIT S

32, Haim Levanon St., (University St.), P.O.Box 39296 Ramat-Aviv, Tel-Aviv 61392, ISRAEL, Phones: 972-3-6408113, 972-3-6428765, Fax: 972-3-6429865
INTERNET: ramot @ ccsg. tau. ac. il

Attorney's Docket No. P306(TelAviv) PATENT

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION OR CIP)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type: *(check one applicable item below)*

__X__ original
____ design
____ supplemental

NOTE: If the declaration is for an international Application being filed as a divisional, continuation or continuation-in-part application do not check next item; check appropriate one of last three items.

____ national stage of PCT

NOTE: If one of the follow 3 items apply then complete and also attach ADDED PAGES FOR DIVISIONAL, CONTINUATION OR CIP.

____ divisional
____ continuation
____ continuation-in-part (CIP)

INVENTORSHIP IDENTIFICATION

WARNING: If the inventors are each not the inventors of all the claims an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PREVENTION OF PROTEIN AGGREGATION

SPECIFICATION IDENTIFICATION

the specification of which: (complete (a), (b) or (c))
(a) __X__ is attached hereto.
(b) ____ was filed on _____ as Serial No. _____
or, if Serial Number not yet known, Express Mail No. _____
and was amended on _____ (if applicable).

NOTE: Amendments filed after the original papers are deposited with the PTO which contain new matter are not accorded a filing date by being referred to in the declaration. Accordingly, the amendments involved are those filed with the application papers or, in the case of a supplemental declaration, are those amendments claiming matter not encompassed in the original statement of invention or claims. See 37 CFR 1.67.

EXHIBIT T

(c) ___ was described and claimed in PCT International Application No. filed on _____ and as amended under PCT Article 19 on (if any).

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

___ In compliance with this duty there is attached an information disclosure statement 37 CFR 1.97.

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(complete (d) or (e))

(d) _X_ no such applications have been filed.

(e) ___ such applications have been filed as follows

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority check item (e), enter the details below and make the priority claim.

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

COUNTRY	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIM UNDER 35 USC 119
			___ YES ___ NO
			___ YES ___ NO
			___ YES ___ NO
			___ YES ___ NO
			___ YES ___ NO

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. (List name and registration number)

E.J. Biskup	18,987		
L.A. Ebling	34,153	H.W. Milton, Jr.	22,180
P.J. Ethington	17,299	J.P. Moran	20,941
J.C. Evans	20,124	O.E. Perry	19,969
R.L. Farris	25,112	R.L. Phillips	20,835
F.J. Fodale	20,824	S.L. Permut	28,388
R.W. Hoffman	33,711	J.E. Shackelford	36,003
K.I. Kohn	30,955	D.J. Simonelli	36,680
J.F. Learman	17,069	J.D. Stevens	35,691
J.K. McCulloch	17,452	C.R. White	20,494

SEND CORRESPONDENCE TO:

Kenneth I. Kohn
Reising, Ethington, Barnard,
Perry & Milton
P.O. Box 4390
Troy, MI 48099-9998

DIRECT TELEPHONE CALLS TO:
(NAME AND TELEPHONE NUMBER)

Kenneth I. Kohn
(810) 689-3554

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application of any patent issued thereon.

SIGNATURE(S)

Full name of sole or first joint inventor: Beka Solomon

Inventor's signature Beka Solomon

Date 7 December 1994 Country of Citizenship Israel

Residence Herzlya Pituah

Post Office Address Hanassi Street 120

Herzlya Pituah

Full name of second joint inventor: if any _____

Inventor's signature _____

Date _____ Country of Citizenship _____

Residence _____

Post Office Address _____

CHECK PROPER BOX(ES) FOR ANY OF THE FOLLOWING ADDED PAGE(S) WHICH FORM A PART OF THIS DECLARATION

____ Signature for third and subsequent joint inventors. Number of pages added _____

____ Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. Number of pages added _____

____ Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR 1.47. Number of pages added _____

* * *

____ Added pages to combined declaration and power of attorney for divisional, continuation, or continuation-in-part (CIP) application.

* * *

____ Authorization of attorney(s) to accept and follow instructions from representative.

* * *

If no further pages form a part of this Declaration then end this Declaration with this page and check the following item

__X__ This declaration ends with this page.

Attorney Docket: P306 (TelAviv)

A S S I G N M E N T

For the sum of One Dollar ($1.00) and other good and valuable consideration, receipt of which is hereby acknowledged, I, **Beka Solomon**, do hereby assign, sell and set over to **RAMOT University Authority for Applied Research & Industrial Development Ltd.**, organized and existing under the laws of Israel and having a place of business in Tel Aviv, Israel, hereinafter referred to as the ASSIGNEE, its successors, assigns or other legal representatives, the entire right, title and interest, domestic and foreign, in and to the inventions and discoveries in

PREVENTION OF PROTEIN AGGREGATION

set forth in the application for United States Letters Patent, Attorney Docket Number P306 (Tel Aviv), executed on even date herewith, including the right of said ASSIGNEE, its successors, assigns, or other legal representatives to make applications and to receive Letters Patent for said inventions and discoveries in any and all foreign countries in its or their own name or names or in my name, at its or their election, and I hereby assign, sell and set over to said ASSIGNEE, its successors, assigns, or other legal representatives, all rights of priority in and to said inventions and discoveries in all countries.

And I hereby agree for myself, my heirs, successors, assigns or other legal representatives to execute any and all papers, including applications for Letters Patent of any and all kinds and in any and all countries, and to perform any and all acts which said ASSIGNEE, its successors, assigns or other legal representatives may deem necessary to secure thereto the rights herein assigned, sold and set over.

And I hereby represent and warrant that I have not granted any rights inconsistent with the rights granted herein.

IN WITNESS WHEREOF, I have hereunto set my hand and seal.

Hananel Kratinsky
Witness

Beka Solomon
BEKA SOLOMON

10, Hagaon Elliahu St.
Witness Address

Date: 7 Dec. 1999

RAMAT-GAN, ISRAEL

EXHIBIT U

FORM 7-4
PATENT

Attorney's Docket Number: P306(TelAviv)

Applicant or Patentee: Beka Solomon

Serial or Patent No:

Filed or Issued: Herewith

For: PREVENTION OF PROTEIN AGGREGATION

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS
(37 CFR 1.9(f) and 1.27(d)--SMALL BUSINESS CONCERN

I hereby declare that I am:

___ the owner of the small business concern identified below:

_X_ an official of the small business concern empowered to sign on behalf of the concern identified below:

Name of Concern: RAMOT-University Authority for Applied Research and Industrial Development Ltd.

Address of Concern: 32 H. Levanon Street, P.O. Box 39296

Tel Aviv 61392 Israel

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement: (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when, either directly or indirectly, one concern controls or has the power to control the other, or a third-party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled:

PREVENTION OF PROTEIN AGGREGATION

By Inventor(s): Beka Solomon

Described in:

_X_ the specification filed herewith.

___ application serial no. _____, filed _____.

___ patent no. _____, issued _____.

EXHIBIT V

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

* NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

NAME:

ADDRESS:

____ Individual ____ Small Business ____ Nonprofit Organization

NAME:

ADDRESS:

____ Individual ____ Small Business ____ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Persons Signing: Hananael Kvatinsky, Assistant R&D Manager, Patents and Technology Transfer

Zvi Shoshan, General Manager

Address: 32 Haim Levanon Street
Tel Aviv 61392 ISRAEL

HANANEL KVATINSKY
Assistant R&D Manager
Patents & Technology Transfer

SIGNATURE: _____ Date: 6-DEC-99

SIGNATURE: _____ Date: 6-Dec.-99

ZVI SHOSHAN
General Manager

Wednesday, December 14, 1994 08:33:55 PM
Message

From: Ken Kohn
Subject: Beka Solomon Application
To: Hananel Kvatinsky
Cc: Jackie Walton

Beka Solomon sent us allot of new material and we will, of course, handle it in due course. Out of all of it, I only have two questions for her that should be straight forward. First, she deleted a couple of limitations from Claim 1 without giving a reason. Could she please explain so we can make sure that the remainder of the application is consistent? Second, we had said that the peptide can mimic the antibody binding site of the monoclonal antibody claimed. She has changed it to the peptide binding to the antigen binding site. This may be a simple change of nomenclature. However, does she mean the binding site of the target molecule or the binding site of the monoclonal antibody? Once we have the answers, we will immediately complete the application and file it.
Regards,
KIK

1180

EXHIBIT W

Thursday, December 15, 1994 01:28:11 PM
Message

From: Ken Kohn
Subject: Beka Solomon
To: Hananel Kvatinsky
Cc: Jackie Walton

Hi. Beka made some major rivisions, asking us to replace the data in the application with the data from two of here papers (J. Biol Chem and Nature). I have done this. It also required replacing all of the figures, their description, and renumbering and collating all of the cited references. The question is, should we go ahead and file it, or should we send you another draft for her review? My opinion is to file it since time is of the essence and amend or CIP it if more data can be added. This seems to be a train of thught thing with her as she perfects her manuscripts and we could keep adding stuff each time we send it to her, which is not bad but makes it impossible to file and keeps runnign uyp the bill for the initial filing. I think that we have the gist of the invention covered. If she says that she really wanted to substitute the data in the papers for all of the data in the draft application (that is what she said but the issue is , is that what she meant?), then certainly let's just file it now. If she says that she needs to see it , then we will E-mail you another draft and it will be filed on Monday, if she get's her comments to us by Sunday or Monday.
Isn't this a blast when you're working on the edge?!!??
By the way, I recieved the disclosure materials from you for we are proceeding with due haste.
Thanks for keeping us busy.
KIK

1180

P-306

EXHIBIT X

9711

TEL AVIV UNIVERSITY אוניברסיטת תל-אביב
GEORGE S. WISE FACULTY OF LIFE SCIENCES
DEPT. OF MOLECULAR MICROBIOLOGY AND BIOTECHNOLOGY
הפקולטה למדעי החיים ע"ש ג'ורג' ס. ויז
המחלקה למיקרוביולוגיה מולקולרית וביוטכנולוגיה

TO:
FAX NO. 6429865
ATTN. HANANEL

DATE:
FROM: Dr Salomon

NO. OF PAGES
(including this page)

OUR FAX No. 972-3-6409407

To Hananel-
please

1) Claim 1 change only lines 11,12 as follows:
... mixing of target molecule with the monoclonal antibodies, exposure to denaturation ...

2) The antibody binding site (epitope) is on the body of the antigen.
We mean to identify the antigen binding site of the antibody which can bind to the antigen at a specific site and to prevent appropriation.

EXHIBIT Y

RAMAT AVIV 69978 TEL AVIV, ISRAEL. TEL. 03-6408111. TELEX: 342171 VERSY IL. CABLE: VERSITY T.A. FAX: 03'6409407 (קווי) רמת-אביב, רמת אביב, תל אביב 69978. טל. 03'6408111. פקס. 03'6409407

TEL AVIV UNIVERSITY אוניברסיטת תל-אביב

GEORGE S. WISE FACULTY OF LIFE SCIENCES
DEPT. OF MOLECULAR MICROBIOLOGY & BIOTECHNOLOGY

הפקולטה למדעי החיים ע"ש ג'ורג' ס. ויז
המחלקה למיקרוביולוגיה מולקולרית וביוטכנולוגיה

2nd November 1994

Dr. Kenneth I. Kohn
Reising, Ethington, Barnard
Perry & Milton
Columbia Center
201 W. Big Weaver Suite 400
P.O.Box 4390
Troy, Michigan 48099
USA

Dear Dr. Kohn,

As agreed during your visit here I am sending you herewith the modifications and supplementary data regarding the patent.

Please contact me directly if you need any assistence. My telephone number is (972-3) 6409711, and fax number (972-3) 6409407.

I am now waiting for your agreement to go ahead and publish the papers and make the necessary application for financial assistance to continue the project.

I do hope you enjoyed your stay in Israel and thank you for all your cooperation.

With best wishes.

Sincerely yours,

Beka Solomon

Beka Solomon, Ph.D.

EXHIBIT Z

RAMAT AVIV, 69978 TEL AVIV, ISRAEL; TEL: 03-6408111 FAX: 972-3-6409407